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Oral Biology

Molecular Techniques
and Applications

Edited by
Gregory J. Seymour
Mary P. Cullinan
Nicholas C. K. Heng

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Oral Biology

Molecular Techniques and Applications

Edited by

Gregory J. Seymour

*Sir John Walsh Research Institute, Faculty of Dentistry,
University of Otago, Dunedin, New Zealand*

Mary P. Cullinan

*Sir John Walsh Research Institute, Faculty of Dentistry,
University of Otago, Dunedin, New Zealand*

Nicholas C.K. Heng

*Sir John Walsh Research Institute, Faculty of Dentistry,
University of Otago, Dunedin, New Zealand*

Editors

Gregory J. Seymour
Sir John Walsh Research Institute
Faculty of Dentistry
University of Otago
310 Great King Street
Dunedin 9016
New Zealand
gregory.seymour@otago.ac.nz

Mary P. Cullinan
Sir John Walsh Research Institute
Faculty of Dentistry
University of Otago
310 Great King Street
Dunedin 9016
New Zealand
mary.cullinan@otago.ac.nz

Nicholas C.K. Heng
Sir John Walsh Research Institute
Faculty of Dentistry
University of Otago
310 Great King Street
Dunedin 9016
New Zealand
nicholas.heng@otago.ac.nz

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Cover illustration: Composite image showing confocal laser scanning microscopy (CLSM) of bacterial invasion of dentinal tubules. Live bacteria fluoresce green/yellow and dead bacteria fluoresce red. Photograph provided by G.R. Tompkins. The CLSM technique is described in Chapter 10.

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Preface

It is generally recognized that the knowledge and research base that underpins dentistry lies in the biological and physical sciences. In this context, the major advances in these sciences over the past two decades have come through the application of molecular biology and nanotechnology. These advances are currently impacting on the diagnosis and treatment of a wide range of human diseases and it is essential that dental research, education, and practice keep pace with this rapidly advancing field. As pointed out by Ford et al. (1):

The definition of disease is also changing. Previously, disease was understood to be the presence of symptoms or of a particular phenotype. With increasing knowledge of the genetic basis of many diseases, this definition is changing to become the presence of a genotype conferring a pre-disposition to clinical symptoms or phenotype (Ford et al. (1)).

This changing definition of disease means that today's undergraduate or graduate student in dentistry (and its related fields) must be in a position not only to acquire new knowledge in the future but also to be able to evaluate the information and apply it in a clinically relevant setting. This naturally positions oral biology as an integral part of any dentally related professional's repertoire of knowledge.

There are as many topics in oral biology as there are the number of sites and microenvironments within the oral cavity. Therefore, it is impossible to cover all aspects in a single volume. Nevertheless, we believe we have compiled a selection of molecular methods and techniques, albeit optimized for particular applications, which can be adapted to a particular organism or area of interest. For ease of presentation, we have divided the volume into three parts. Section I describes techniques applicable to the study of saliva, the fluid that is exquisitely unique to the oral cavity. Saliva is not only one of the first lines of defense against microbial invaders but also a rich source of biomolecules for study at the molecular level, which may lead to the identification of susceptibility to particular diseases. Among the techniques presented are those pertaining to the preparation of salivary samples for proteomic and genetic purposes. Section II is devoted to the study of the microbial inhabitants that share the oral cavity with us, and the methods provided will allow the study of the oral microbiota as a whole (microbial diversity and biofilms) or only of select members (microbial physiology or natural genetic transformation). Furthermore, techniques to identify putative immunogenic proteins from microbial pathogens as well as ways of producing such proteins in heterologous hosts allow the reader to examine the influence of single biomolecules on the host response. Lastly, Section III provides a range of protocols that facilitate assessment of the molecular behavior of oral cells and tissues in health and during disease progression. The present age that we live in is full of nanotechnological advances, and sophisticated instruments capable of high-throughput sample processing, especially for DNA sequencing and microarray applications, are available and increasing in popularity. Hence, some of the techniques presented in this volume potentially generate an enormous quantity of data. As we feel that it is just as important to be able to analyze and interpret these data as it is in obtaining them in the first place, certain chapters include sections on bioinformatic analyses.

This volume will be a useful resource not only to the new researcher but also to the seasoned laboratory veteran including cell biologists, microbiologists, and any researcher intent on delving into the exciting world of oral biology.

Gregory J. Seymour
Mary P. Cullinan
Nicholas C. K. Heng

Reference

1. Ford, P. J., Seymour, G. J. et al. (2008) Adapting to changes in molecular biosciences and technologies. *Eur. J. Dent. Educ.* **12**(Suppl 1), 40–47.

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Contributors

- JACQUELINE ABRANCHES • *Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, USA; Center for Oral Biology, University of Rochester Medical Center, Rochester, NY, USA*
- JANIK ADRIAANSEN • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
- ABDUL AZIZ • *School of Dentistry, Sir John Walsh Research Institute, University of Otago, Dunedin, New Zealand*
- P. MARK BARTOLD • *Dental School, Colgate Australian Clinical Dental Research Centre, University of Adelaide, Adelaide, Australia*
- BRUCE J. BAUM • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
- ROBERT A. BURNE • *Department of Oral Biology, University of Florida College of Dentistry, Gainesville, FL, USA*
- RICHARD D. CANNON • *Department of Oral Sciences, School of Dentistry, University of Otago, Dunedin, New Zealand*
- PO-CHUN CHANG • *Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA*
- KENNETH CHONG • *Department of Oral Sciences, School of Dentistry, University of Otago, Dunedin, New Zealand*
- PATTY CHOU • *Faculty of Dentistry, Sir John Walsh Research Institute, University of Otago, Dunedin, New Zealand*
- JIN CHUNG • *Center for Molecular Microbiology, University of Florida College of Dentistry, Gainesville, FL, USA; Department of Oral Biology, University of Florida College of Dentistry, Gainesville, FL, USA*
- ANA P. COTRIM • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
- RYAN T. DEMMER • *Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA*
- PAULINE J. FORD • *School of Dentistry, The University of Queensland, Brisbane, Australia*
- LEA M. FRANCO • *Department Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA*
- WILLIAM V. GIANNOBILE • *Department Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA; Department Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, MI, USA; School of Dentistry, Michigan Center for Oral Health Research, University of Michigan, Ann Arbor, MI, USA*
- CORINNE M. GOLDSMITH • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
- STAN GRONTHOS • *Mesenchymal Stem Cell Group, Division of Hematology, Institute of Medical and Veterinary Science/Hanson Institute, Adelaide, Australia*
- STEPHEN M. HAMLET • *School of Dentistry and Oral Health, Griffith University, Southport, Queensland, Australia*

- MARTIN HANDFIELD • *Center for Molecular Microbiology, University of Florida College of Dentistry, Gainesville, FL, USA*
- NICHOLAS C.K. HENG • *Faculty of Dentistry, Sir John Walsh Research Institute, University of Otago, Dunedin, New Zealand*
- BRADLEY STEPHEN HENSON • *College of Dental Medicine, Western University of Health Sciences, Pomona, CA, USA*
- ANN R. HOLMES • *Department of Oral Sciences, School of Dentistry, University of Otago, Dunedin, New Zealand*
- SHEN HU • *School of Dentistry, University of California Los Angeles, Los Angeles, CA, USA; Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, CA, USA*
- MICHAEL J. HUBBARD • *Department of Pharmacology, University of Melbourne, Melbourne, Australia; Department of Paediatrics, Royal Children's Hospital, University of Melbourne, Melbourne, Australia*
- HARUE ITO • *Laboratory of Periodontology and Immunology, Department of Oral Health and Welfare, Center for Transdisciplinary Research, Niigata University, Niigata, Japan*
- JIANG JIANG • *School of Dentistry, University of California Los Angeles, Los Angeles, CA, USA; Research and Diagnostic Systems, Inc*
- MORITZ KESCHULL • *Division of Periodontics, Section of Oral and Diagnostic Sciences, College of Dental Medicine, Columbia University, New York, NY, USA*
- JEW C. KON • *Department of Pharmacology, University of Melbourne, Melbourne, Australia; Department of Paediatrics, University of Melbourne, Melbourne, Australia*
- HYUN KOO • *Department of Microbiology and Immunology, Center for Oral Biology, University of Rochester Medical Center, Rochester, NY, USA; Eastman Department of Dentistry, University of Rochester Medical Center, Rochester, NY, USA*
- JENS KRETH • *Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
- ERWIN LAMPING • *Department of Oral Sciences, School of Dentistry, University of Otago, Dunedin, New Zealand*
- JOSÉ A. LEMOS • *Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY, USA; Center for Oral Biology, University of Rochester Medical Center, Rochester, NY, USA*
- KARL M. LYONS • *Department of Oral Rehabilitation, School of Dentistry, University of Otago, Dunedin, New Zealand*
- JONATHAN E. MANGUM • *Department of Pharmacology, University of Melbourne, Melbourne, Australia*
- ROBERT E. MARQUIS • *Department of Microbiology and Immunology, Center for Oral Biology, University of Rochester Medical Center, Rochester, NY, USA*
- ANDREW MCNAUGHTON • *Department of Anatomy and Structural Biology, Otago Centre for Confocal Microscopy, University of Otago, Dunedin, New Zealand*
- TRUDY J. MILNE • *Faculty of Dentistry, Sir John Walsh Research Institute, University of Otago, Dunedin, New Zealand*
- KRZYSZTOF MROZIK • *Mesenchymal Stem Cell Group, Division of Hematology, Institute of Medical and Veterinary Science/Hanson Institute, Adelaide, Australia*
- KATJA NÄRHI • *Institute of Biotechnology, University of Helsinki, Helsinki, Finland*
- VISWANATHAN PALANISAMY • *College of Dental Medicine, Medical University of South Carolina, Charleston, SC, USA*

- PANOS N. PAPAPANOU • *Division of Periodontics, Section of Oral and Diagnostic Sciences, College of Dental Medicine, Columbia University, New York, NY, USA*
- CHAN HO PARK • *Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA*
- DIKESH PARMAR • *School of Dentistry, Sir John Walsh Research Institute, University of Otago, Dunedin, New Zealand*
- PAUL PAVLIDIS • *Centre for High-throughput Biology and Department of Psychiatry, University of British Columbia, Vancouver, BC, Canada*
- GAIA PELLEGRINI • *Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA*
- PAOLA PEREZ • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
- FERNANDA CRISTINA PETERSEN • *Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway*
- SEN RONG QI • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
- FENGXIA QI • *College of Dentistry, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA*
- ISABELA N. RÔÇAS • *Department of Endodontics and Molecular Microbiology, Estácio de Sá University, Rio de Janeiro, RJ, Brazil*
- ALEXANDRE S. ROSADO • *Institute of Microbiology Prof. Paulo de Góes, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
- ANNE M. ROWZEE • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
- MITSUO SAKAMOTO • *Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan*
- ANNE AAMDAL SCHEIE • *Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway*
- YANG-JO SEOL • *Department of Periodontics and Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA*
- SONGTAO SHI • *University of Southern California, Los Angeles, CA, USA*
- JOSÉ F. SIQUEIRA JR. • *Molecular Microbiology Laboratory, Department of Endodontics, Faculty of Dentistry, Estácio de Sá University, Rio de Janeiro, RJ, Brazil*
- JO-ANN L. STANTON • *Department of Anatomy and Structural Biology, Otago High-Throughput DNA Sequencing Facility, University of Otago, Dunedin, New Zealand*
- KOICHI TABETA • *Center for Transdisciplinary Research, Niigata University, Niigata, Japan*
- IRMA THESLEFF • *Developmental Biology Program, Institute of Biotechnology, University of Helsinki, Helsinki, Finland*
- GEOFFREY R. TOMPKINS • *School of Dentistry, Sir John Walsh Research Institute, University of Otago, Dunedin, New Zealand*
- SHANNON M. WALLET • *Department of Periodontology, University of Florida College of Dentistry, Gainesville, FL, USA; Center for Molecular Microbiology, University of Florida College of Dentistry, Gainesville, FL, USA; Department of Oral Biology, University of Florida College of Dentistry, Gainesville, FL, USA*
- DAVID T. WONG • *Division of Oral Biology and Oral Medicine, University of California Los Angeles, Los Angeles, CA, USA; Dental Research Institute, University of California Los Angeles, Los Angeles, CA, USA; School of Dentistry, University of California*

Los Angeles, Los Angeles, CA, USA; Department of Craniofacial Biology, University of California Los Angeles, Los Angeles, CA, USA; Jonsson Comprehensive Cancer Center, University of California Los Angeles, Los Angeles, CA, USA; Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA, USA; Henry Samuel School of Engineering and Applied Science, University of California Los Angeles, Los Angeles, CA, USA

KAZUHISA YAMAZAKI • *Center for Transdisciplinary Research, Niigata University, Niigata, Japan; Laboratory of Periodontology and Immunology, Department of Oral Health and Welfare, Niigata University, Niigata, Japan*

CHANGYU ZHENG • *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*

Section I

Saliva Studies

Chapter 1

Gene Therapy of Salivary Diseases

**Bruce J. Baum, Janik Adriaansen, Ana P. Cotrim,
Corinne M. Goldsmith, Paola Perez, Senrong Qi,
Anne M. Rowzee, and Changyu Zheng**

Abstract

For many years, our laboratory has been developing gene transfer approaches for salivary gland disorders that currently lack effective therapy. The purpose of this chapter is to describe key methods used in this developmental process. Specifically, we focus on one clinical condition, irradiation-induced salivary hypofunction, and address the choice of transgene and vector to be used, the construction of recombinant viral vectors, how vector delivery is accomplished, and methods for assessing vector function in vitro and in an appropriate animal model.

Key words: Gene therapy, salivary glands, adenovirus, adeno-associated virus, radiation damage, salivary hypofunction.

1. Introduction

There are two major disorders that lead to the irreversible loss of salivary gland function: (i) irradiation damage that occurs during the course of treatment for a head and neck cancer and (ii) the autoimmune exocrinopathy Sjögren's syndrome. Both disorders are fairly common. In 2006, there were more than 40,000 new cases of head and neck cancer diagnosed in the United States, accounting for ~3% of all malignancies (1), with ~500,000 people affected worldwide annually. The treatment for most such patients, in industrialized societies, includes surgery and irradiation $\pm$ chemotherapy. Sjögren's syndrome has a prevalence of ~0.5–1%, making it the second most common rheumatic disease after rheumatoid arthritis (2). Although the etiologies of these two disorders are dramatically different, both conditions result in the loss of salivary acinar cells, the only cell type that normally

secretes the fluid component of saliva. With both conditions, the predominant remaining epithelial cells are of duct origin and incapable of fluid secretion. Patients lacking saliva suffer considerable morbidity, including dysphagia, oral infections, delayed mucosal healing, and considerable pain and discomfort.

If patients in either disease group have a reasonable mass of acinar cells remaining, treatment with sialogogues (salivary stimulants, e.g., pilocarpine, civemaline) can be beneficial. However, for patients who lack most or all salivary acinar cells, currently there is no suitable treatment available, a situation that provided the impetus for our beginning to explore the use of *in vivo* gene transfer (gene therapy). It is also important to recognize a key difference in these two conditions: irradiation-induced salivary hypofunction is a localized gland problem. While this condition certainly leads to some systemic concerns (e.g., dysphagia, infections), a primary treatment for it needs only be targeted to the damaged gland. Furthermore, the pathologic etiology, i.e., the radiation treatment, is time limited. A patient presenting with irradiation-induced salivary hypofunction was treated with radiotherapy in the past and is without any ongoing active disease process. Conversely, a patient with Sjögren's syndrome experiences a systemic autoimmune disease, albeit one typically having salivary glands as a major target organ. Localized salivary gland gene therapy for a patient with Sjögren's syndrome can address their salivary hypofunction (3), but in all likelihood, at least for the present, will have no beneficial effects on the systemic disease process. Additionally, it is important to recognize that Sjögren's syndrome patients show continuous disease activity, e.g., the presence of serum autoimmune markers. Based on its more localized nature and the absence of an active disease process, irradiation-induced salivary hypofunction is a disorder more readily treatable by salivary gland gene therapy. It also lends itself to a more useful presentation for a chapter such as this. Hence, the focus of this chapter is only on the "repair" of irradiation-induced salivary hypofunction.

As with the development of a therapy for any disease condition, an essential initial element is to have a good understanding of the physiology of the normal target tissue, in addition to a good understanding of the pathophysiological situation. Fortunately, our laboratory has such a background, as a result of working many years in the salivary gland field (4). Based on that understanding, we decided that surviving duct cells in the irradiated gland were capable of generating an osmotic gradient into the gland lumen, but needed water permeability pathways to allow fluid to follow this gradient (5). Accordingly, the gene of choice for our approach was aquaporin-1 (AQP1), the first described water channel (6). Given that salivary epithelial cells are slowly dividing post-mitotic cells, it was appropriate to employ

non-integrating vectors for the actual gene transfer. Accordingly, we have used recombinant serotype 5 adenoviral (rAd5) and serotype 2 adeno-associated viral (rAAV2) vectors (7). Each vector type has distinct advantages. For example, rAd5 vectors are relatively easy to produce, lead to high transgene expression, but induce a potent immune response that renders the expression transient (<2–4 weeks). rAAV2 vectors elicit a modest immune response and generally lead to low, but stable, transgene expression; however, they are more difficult to produce. We initially used rAd5 vectors for proof-of-concept studies in both animal models and humans and now are working on developing a long-lived therapeutic correction of irradiation-induced salivary hypofunction by using a rAAV2 vector. Herein, we will describe the production of both vector types.

1.1. Recombinant Serotype 5 Adenoviral Vector Production

Adenoviruses are non-enveloped, double-stranded DNA viruses. There are more than 50 human adenoviral serotypes. A common adenoviral gene transfer vector is based on adenovirus serotype 5 and we typically use an E1 gene-deleted, replication-deficient rAd5 vector in our studies. Importantly, these vectors can efficiently transduce salivary acinar and duct cells (8). To construct, propagate, and purify an E1 deleted rAd5 vector, various products and services from different companies can be used, including Microbix Biosystems (Toronto, Ontario, Canada), Clontech (Mountain View, CA), Cell Biolabs, Inc. (San Diego, CA), Invitrogen (Carlsbad, CA), and Stratagene (La Jolla, CA). In the first-generation (simplest) rAd5 vectors, the E1 gene is deleted. This gene encodes the E1a and E1b proteins, which are required for the replication of a wild-type serotype 5 adenovirus. This gene deletion creates space in the vector genome to insert a foreign gene or cDNA (the transgene).

Currently, there are two principal ways to generate E1 deleted rAd5 vectors: using a two-plasmid co-transfection method with eukaryotic (293) cells and an *E. coli*-based system (from Stratagene). Herein, we will describe the two-plasmid co-transfection method to generate a rAd5 vector, as this is the method we routinely use. In the two-plasmid system, one plasmid is termed a shuttle vector (the one used in our laboratory is pACCMV-pLpA) and can express in both prokaryotic and eukaryotic cells. This plasmid has a cytomegalovirus (CMV) promoter, a multiple cloning site, and a SV40 polyadenylation signal that can be placed in the E1 region between map units 1.3 and 9.1 of the adenoviral genome. This plasmid also contains 455 base pairs of serotype 5 adenoviral sequence (0.0–1.3 map units) upstream of the CMV promoter and 3,039 base pairs of serotype 5 adenoviral sequence (9.3–17.0 map units) downstream of the SV40 polyadenylation signal. The second plasmid is pJM17, and it provides most of the serotype 5 adenoviral sequence except for the E1

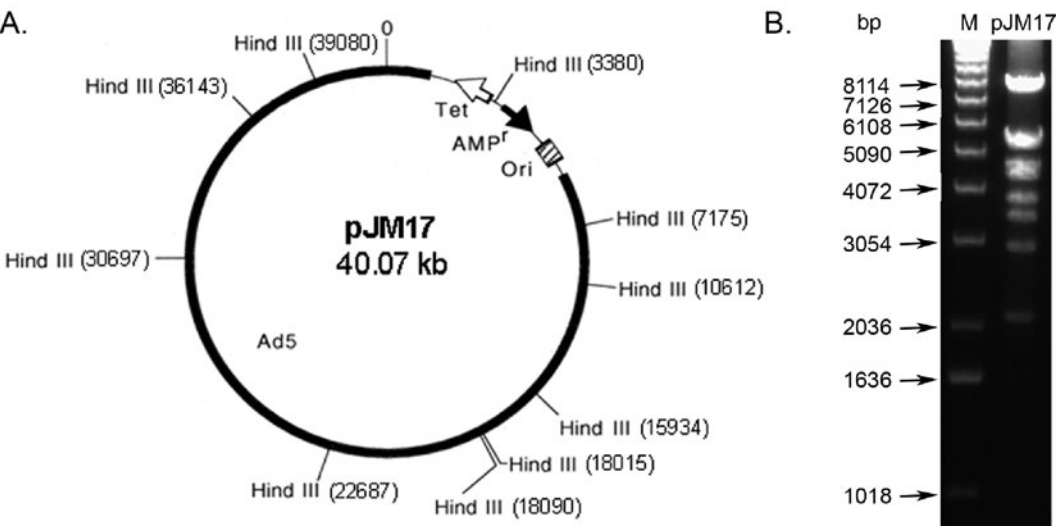


Fig. 1.1. **A.** Map of pJM17 plasmid showing results of Hind III digestion. **B.** Gel showing Hind III digestion results. There are 10 *Hind*III restriction endonuclease sites in the pJM17 plasmid. After digestion, it yields 10 fragments (8,010, 5,446, 5,322, 4,597, 4,370, 3,795, 3,437, 2,937, 2,081, and 75 bp). Eight of these fragments from the Hind III digested pJM17 are readily visualized as bands on 1% agarose gels after electrophoresis.

gene and a small part of the E3 gene region. The pJM17 plasmid (Fig. 1.1) contains overlapping sequences with the serotype 5 adenoviral sequences found in pACCMV-pLpA, which permit homologous exchange when they are co-transfected into 293 cells, a human embryonic kidney cell line (9). The 293 cells stably express the E1 proteins necessary to allow replication of the constructed E1-deleted rAd5 vector (10).

1.2. Recombinant AAV Serotype 2 Vector Production

The production of rAAV2 can be generally accomplished with either of two methods: the use of stable packaging cell lines or transient transfection of production cell lines. Both methods require three genetic elements: (i) the “recombinant AAV2 genome,” consisting of the wild-type AAV2 inverted terminal repeats (ITRs) framing the expression cassette. The ITRs contain the *cis* sequence information needed for replication and encapsidation; (ii) the genes coding for the replication and structural proteins (rep and cap, respectively), expressed in *trans*; (iii) the required helper functions naturally encoded by a helper virus such as adenovirus.

To generate rAAV2 using stable packaging cell lines, the expression cassette is stably transfected together with rep and cap genes into a cell line (e.g., HeLa cells). These cells can be stored and expanded for rAAV2 production, which then only requires a wild-type Ad5 infection. This method is easily scalable and thus very suitable for large-scale production in vector core facilities. However, this process is quite laborious if used for only a few

preparations. Also, stable transfection of the rep and cap genes, encoding toxic proteins, can be technically challenging. Contamination with wild-type Ad5 can be a problem due to the infection of stably transfected cell lines with Ad5 to start the production process, but new purification methods to overcome this are being developed.

In contrast, the relatively undemanding transient transfection method is ideally suited for mid-scale production and is what we use in our laboratory. This classical rAAV2 production method is based on a *trans*-complementary co-transfection of plasmids containing the required genetic elements into an appropriate target cell (we use Naut cells; *see* below). It allows flexible and quick rAAV2 production using a straightforward protocol described below (3). The expression cassette is present in the pAAV plasmid flanked by ITRs (11). For optimal yield and convenience, we use a packaging/helper plasmid, pDG, which contains the rep and cap sequences, and also the wild-type Ad5 helper genes. With this method, the typical yield after CsCl centrifugation and subsequent dialysis is between 1×10^{12} and 5×10^{12} total particles, as determined by QPCR.

1.3. Cannulation of Salivary Glands

Among the most common target organs for in vivo gene therapy are the liver, lung, and muscle. The liver and lung have considerable metabolic activity and bulk protein production, but are not easy to access. Muscle, while easy to access for vector delivery, is not a tissue known for protein production and secretion. Salivary glands present an attractive target with some particular advantages for in vivo gene transfer. First, they are easy to access, as the ductal orifices of the glands open into the mouth and typically can be visualized and cannulated without significant complications. Indeed, contrast imaging of cannulated glands (sialography) is a routine clinical diagnostic procedure. Second, although salivary glands are densely packed, almost all acinar and ductal cells have their apical membranes directly in contact with the oral cavity. Third, the majority of salivary parenchymal cells (acinar) are capable of producing high amounts of protein for both exocrine and endocrine secretion. Therefore, a transgene can be targeted to a large number of protein-producing cells with little difficulty.

1.4. Assessing Functional Response (Saliva Collection)

After delivery of the transgene of interest into salivary glands using a viral vector, it is essential to assess the function of the transgene. For the purpose outlined in this chapter, the key biological function to measure is salivary flow rate. We typically collect whole saliva from the floor of the mouth in rodent experiments (Fig. 1.2), although saliva can be collected from individual glands, as well. Whole saliva is much easier and is better for animal survival, which is particularly important if you are conducting long-term studies with multiple sample collections.



Fig. 1.2. Figure of mouse during the whole saliva collection procedure. Note the positioning of the capillary tube draining the saliva from the floor of the mouth into a pre-weighed Eppendorf tube.

1.5. Animal Models

An animal model provides an *in vivo*, non-human experimental tool that mimicks a disease or injury that is similar to a human condition. The use of animal models allows the investigation of pathology and pathogenesis in ways that are impossible in a human patient. Thus, animal models provide scientists with a rich experimental resource, but also require high ethical behavior and great care when used. All animal studies should be conducted using a detailed, written experimental protocol that is reviewed by an appropriate and independent oversight body to ensure that minimal animal pain or discomfort occurs, and that the study is scientifically valid.

1.5.1. Irradiation of Salivary Glands

Irradiation in animal models has long been used to mimic salivary hypofunction in patients who receive irradiation to treat head and neck cancer. Many different animals can be used and our group has employed rhesus monkeys, miniature pigs, rats, and mice at various times and for various purposes as pre-clinical models of salivary gland irradiation damage. Mice are advantageous, as they are small, easy to handle, and inexpensive. Thus, for the purpose of this chapter, we will provide details on irradiating mice and the particular procedures we use. Anyone who is contemplating studying irradiated animals, of whatever species, is urged to consult radiation biology experts at your institution.

For our studies, the mice are 7–10 weeks old (young adults) and typically weigh 20–30 g. We irradiate salivary glands by placing each animal into a specially built Lucite jig in such a way that the animal can be immobilized without the use of anesthesia. The use of anesthesia can change the sensitivity to irradiation, so if anesthesia must be used, it must be used in all experimental and control groups for proper comparison. Additionally, the jig is fitted with a Lucite cone that surrounds the animal's head and prevents head movement during the radiation exposure (*see Fig. 1.3*). For single-dose irradiation experiments in mice, we use a 15 Gy dose (12). Single-dose studies are very convenient, but they do not fully mimic clinical irradiation protocols. Clinically, patients receive fractionated doses, commonly $\sim 1.5\text{--}2.5$ Gy/day 5 days/week, for up to 6 weeks. For our studies in mice we utilize an adapted fractionated-dose scheme, five daily fractions of 6 Gy. This is more convenient than the human regimen, yet one that leads to significant salivary hypofunction (13). Radiation is delivered to the animal's head with a Therapax DXT300 X-ray irradiator (Precision X-ray, Inc., North Branford, CT) using 2.0 mm Al filtration (300 KVp) at a dose rate of 1.9 Gy/min. The single-dose and fractionated-dose schemes described achieve

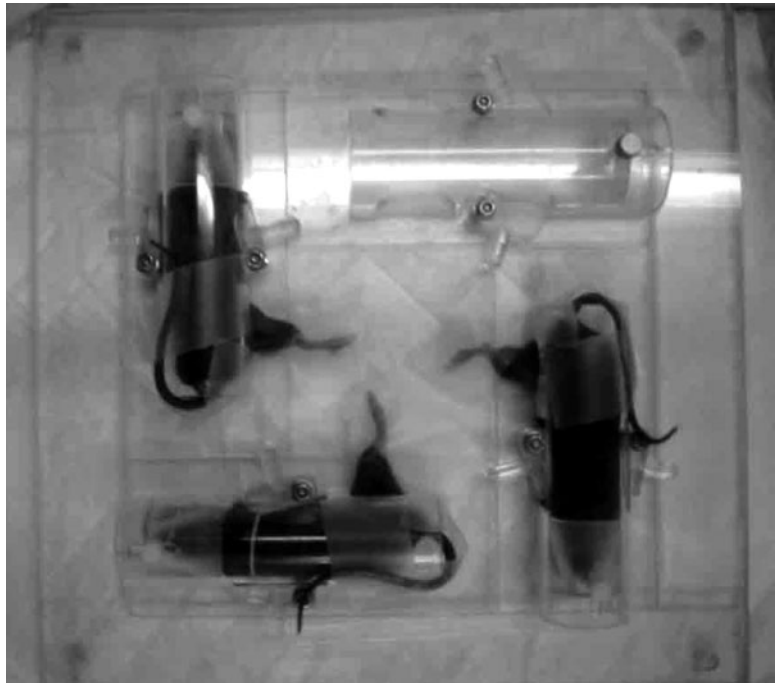


Fig. 1.3. Figure of three mice in the specially made Lucite jig prior to irradiation. The animals are immobilized without the use of anesthesia. Note that the jig is fitted with a Lucite cone that surrounds the animal's head and prevents head movement during the radiation exposure.

comparable salivary hypofunction, resulting in ~50–60% reduction when measured 8 weeks after irradiation was completed (12, 13). Immediately after radiation, animals are removed from the Lucite jig and housed (four animals per cage) in a climate- and light-controlled environment and allowed free access to food and water.

2. Materials

2.1. Materials for Generating a rAd5

1. 293 cell line (Microbix)
2. IMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin G, and 100 µg/mL streptomycin for 293 cell line
3. pJM17 plasmid (Microbix Biosystems, Toronto, Canada)
4. Calcium Phosphate Transfection Kit (Invitrogen)
5. Ultra-clear centrifuge tubes (Beckman Coulter, Fullerton, CA)
6. Cesium chloride (CsCl) gradients (1.25, 1.33 and 1.4 mg/mL) for rAd5 purification. CsCl (Invitrogen) is dissolved in TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 25 mM Tris-HCl, pH 7.4)
7. SW41 rotor (Beckman Coulter) or equivalent
8. PIERCE Slide-A-Lyzer dialysis cassette (ThermoFisher Scientific, Rockford, IL)
9. Virus dialysis buffer: 100 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, and 10% (v/v) glycerol
10. Virus dilution buffer: 5 mM MgCl₂, 20% (v/v) glycerol, 10 mM Tris-HCl, pH 7.4
11. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA)
12. ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA)

2.2. Materials for Generating a rAAV2 Vector

1. Naut 293 cells (Microbix Biosystems)
2. 150 mm plates
3. Dulbecco's Modified Eagle's Medium (DMEM), low glucose (1 g/L D-glucose) medium (Gibco, Carlsbad, CA), 100 U/mL penicillin G, 100 µg/mL streptomycin, 2 mM glutamine, 10% fetal bovine serum)
4. pAAV plasmid containing expression cassette and ITRs
5. pDG plasmid (PlasmidFactory GmbH & Co, Bielefeld, Germany (14))

6. Solution A (stock for fifty 150 mm plates):
 - a. Water: 39.15 mL
 - b. 120 mM dextrose: 5.1 mL
 - c. 10× HBS (Hepes-buffered saline): 5.0 mL
 - d. 1 N NaOH: 0.75 mL
 - e. 140 mM NaPO₄: 1.0 mL
pH should be 7.01–7.04
7. Solution B (per five 150 mm plates):
 - a. 4.3 mL water
 - b. Add plasmid DNA:
 - 90 µg pDG
 - 30 µg pAAV-transgene
 - c. Add 100 µL of 2 M CaCl₂ and vortex
 - d. Add 500 µL of 2 M CaCl₂ and vortex
8. TD buffer:
 - a. 140 mM NaCl
 - b. 5 mM KCl
 - c. 0.7 mM K₂HPO₄
 - d. 25 mM Tris-HCl
Set pH to 7.4
9. Benzonase (also called endonuclease from Sigma-Aldrich, St. Louis, MO)
10. 50 mL conical tubes
11. CsCl
12. Beckman Ultra-Clear Tube (Beckman)
13. Ultracentrifuge and Beckman SW41 Ti rotor and buckets
14. Refractometer
15. “Butterfly needle” (21G ³/₄ Vacutainer; Becton Dickinson)
16. Several Eppendorf microcentrifuge tubes
17. Dialysis cassette (Pierce)
18. Saline
19. 10× Citric saline:
 - a. 50 g KCl
 - b. 22 g Na citrate
 - c. Water to 500 mL

**2.3. Materials
for Delivery of Vector
to Rodent
Submandibular
Glands**

1. Rats or mice
2. Rack made by us for oral cavity display (**Fig. 1.4**)
3. Spring for spreading cheeks (made from paper clip)
4. Cotton to hold back tongue

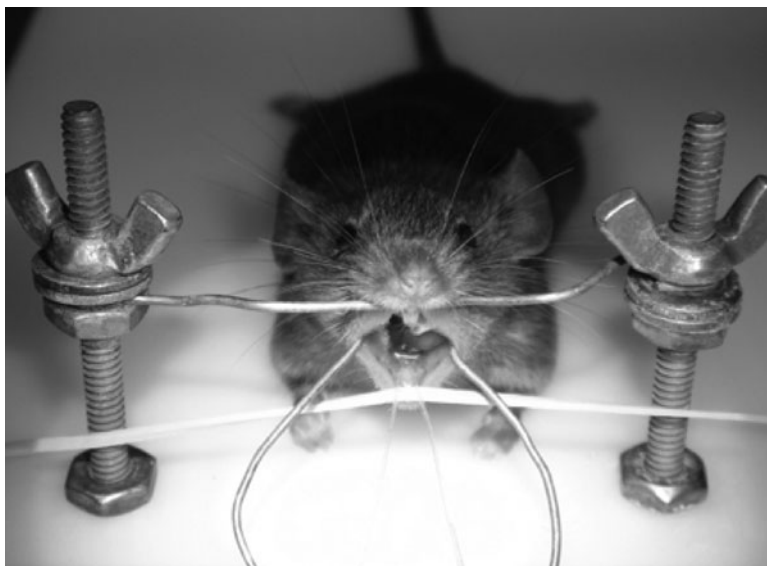


Fig. 1.4. Figure of mouse with cannulae inserted into the orifices of Wharton's duct (submandibular gland) prior to vector delivery (note needle and syringe attached to each cannula are out of the plane of this picture). Place the upper jaw of the mouse on a wire, i.e., teeth over the wire, and pull the lower jaw down with rubber band onto rack. Finally, expand cheeks with wire spring made from a bent paper clip.

5. Microfine IV needle BD insulin syringe 0.3 cc, 28-gauge needle
6. Preformed PE10 tubing (Intramedic PE10 polyethylene tubing, Clay Adams Brand, Becton Dickinson), heated and pulled to make cannulae (*see Note 1*)
7. Scalpel blade to bevel cannulae
8. Binocular dissecting microscope
9. Krazy glue (ethyl cyanoacrylate; Elmer's Products, Inc., Columbus, OH)
10. Curved forceps with very fine tip
11. Ketamine (Ketaject, 100 mg/mL, Phoenix Pharmaceutical, St. Joseph, MO)
12. Xylazine (XYLA-JECT, 20 mg/mL; Phoenix Pharmaceutical)
13. Atropine (Sigma-Aldrich, A0257) made by dissolving powder in water at 0.5 mg/mL (can be stored in aliquots at -20°C)

2.4. Materials for Mouse Whole Saliva Collections

1. Heparinized Microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA)
2. Pilocarpine (Sigma-Aldrich)

3. Ketamine (100 mg/mL, Phoenix Scientific) and xylazine (20 mg/mL, Phoenix Scientific).

3. Methods

3.1. Methods for Generating a rAd5

The following protocol is what we use routinely to produce an E1-deleted rAd5 vector.

1. Insert transgene of interest into multiple cloning site of pACCMV-pLpA using conventional molecular cloning methods to create the shuttle vector.
2. Make a high-quality preparation of shuttle vector.
3. Make a high-quality preparation of the plasmid pJM17 (*see Fig. 1.1; Note 2*).
4. Grow 293 cells in IMEM.
5. Co-transfect pJM17 and the shuttle vector into 293 cells by calcium phosphate co-precipitation using the Calcium Phosphate Transfection Kit (Invitrogen). Add 15 μg of pJM17, 5 μg of shuttle vector, 36 μL of 2 M CaCl_2 and use double-distilled H_2O to bring volume to 300 μL in a 14 mL polypropylene tube, and then mix well. Next, quickly add 300 μL 2 $\times$ Hepes-buffered saline and bubble with an automatic pipet for 1 min, and then incubate at room temperature for 30 min until a fine precipitate is formed. Add the entire mixture to a 100 mm plate of 293 cells and gently rotate the plate to mix well. Put the plate back into the tissue culture incubator and replace the growth medium every 2 days. To increase the chances of successful vector formation, usually four to five plates of cells are co-transfected. After ~ 10 –12 days, plaques (holes in the 293 monolayer) can be observed, which indicate the replication of the recombinant adenovirus. Continually, and carefully, replace the medium until $\sim 50\%$ of 293 cells are lysed. Harvest the entire plate (293 cells and growth medium) by pipetting the growth medium present to dislodge the cell monolayer, i.e., do not use trypsin, and collect into a 50 mL conical test tube.
6. Completely lyse the 293 cells by freezing (dry ice) and thawing (37°C water bath) five times to release rAd5 vector from the cells. Centrifuge at $\sim 700g_{\text{ave}}$ for 5 min. Transfer supernatant [crude viral lysate (CVL)] to a new 50 mL tube. Use 50 μL of the CVL to infect one well of 293 cells (10^5 cells/well in 12-well plate). Measure the recombinant protein production by an appropriate method (e.g.,

by Western blot, ELISA, enzyme assay) in the lysate or medium of these cells after 24 h to confirm that the CVL contained functional virus.

7. To make a large-scale preparation of the recombinant rAd5 vector, use the above CVL to transduce three fresh 100 mm plates of 293 cells. Harvest the plates of cells at day 3. Freeze and thaw five times as above, combine, and again centrifuge to collect supernatant. Use this CVL to transduce twenty 150 mm plates of 293 cells. Harvest the cells at day 3, centrifuge the harvested cells, and aspirate all but ~6 mL of the medium. Use the remaining 6 mL to re-suspend the cell pellet. Freeze and thaw the cell suspension five times and then centrifuge at $\sim 1,600g$ for 10 min.
8. Make the first CsCl gradient by placing 2.5 mL of density 1.25 CsCl in sterile ultra-clear centrifuge tubes and then slowly underlay with 2.5 mL of density 1.40 CsCl. Transfer the CVL supernatant to the top of the CsCl gradient. Spin the tubes in a SW41 rotor or equivalent at $\sim 151,000g_{ave}$, 22°C for 1 h (balance carefully with culture medium or phosphate-buffered saline). Following centrifugation, clean the outside of the tube with 70% alcohol and then use a 21 gauge needle and syringe to poke through the side of the tube. Collect the lower opalescent band, which contains the rAd5 vector. Place 8 mL of density 1.33 CsCl into sterile ultra-clear centrifuge tubes and transfer the vector band to this second CsCl gradient. Spin the tubes in a SW41 rotor at $\sim 151,000g_{ave}$, 22°C for 18 h (again balancing carefully). As above, clean the outside of the tube with 70% alcohol, and again use a 21-gauge needle and syringe to collect the opalescent band and add glycerol to 10% (*see Note 3*).
9. Transfer the vector into a PIERCE Slide-A-Lyzer dialysis cassette and dialyze two times in 500 mL dialysis buffer for 30 min, and then in 1 L fresh dialysis buffer for 1 h, three times. Remove the vector suspension from the dialysis cassette and aliquot in sterile Eppendorf tubes at $\sim 100\ \mu\text{L}/\text{tube}$ and store at -80°C .
10. Use real-time PCR (QPCR) to titer the rAd5 vector with primers from the E2 region of adenovirus, E2q1 (5'-GCAGAACCAACAGCACAGTGT-3'), and E2q2 (5'-TCCACGCATTTTCCTTCTAAGCTA-3'). Titers are expressed as particles per milliliter. The plasmid pACCMV-pLpA can be used as a standard for QPCR, with 1 μg of the plasmid ($\sim 10,132$ base pairs) being equivalent to 9.0×10^{10} molecules. Standard curves are established from 10^1 molecules to 10^8 molecules of shuttle vector, and rAd5 vectors are tested at three dilutions over a 100-fold

range. These QPCR assays are typically carried out with the SYBR Green PCR Master Mix from Applied Biosystems using an ABI prism 7700 Sequence Detector, with the following PCR parameters: initial denaturation 95°C for 2 min followed by 40 cycles of denaturation 95°C for 1 min, annealing at 95°C for 15 s, and extension at 60°C for 1 min.

3.2. Methods for Generating a rAAV2 Vector

1. Culture thirty 150 mm plates of Naut 293 cells in DMEM medium at $\sim 6 \times 10^5$ cells/plate.
2. Incubate cells in DMEM overnight (reaching 30% confluency).
3. Transfection:
 - a. Make a stock (for 50 plates) of “solution A”; store at room temperature.
 - b. Make “solution B” fresh each time (per five plates).
4. Add 5.1 mL of solution A in a 50 mL tube (per five plates).
5. Place a 2 mL pipette in solution A and continuously bubble while adding solution B drop by drop.
6. Incubate until a white precipitate is seen (at least 30 min), vortex and add 2.0 mL/plate, drop by drop while gently swirling the plate.
7. After 48 h, remove medium and rinse each plate with 5.0 mL 1× citric saline (dilute 10× citric saline with water); incubate 5 min.
8. Using 10 mL of TD buffer, detach the cells from the plate, and use the same 10 mL of TD to harvest five plates.
9. With another 10 mL of TD buffer, rinse all plates.
10. Put TD buffer containing the cell suspension from these five plates into a 50 mL tube. Repeat steps 3–10 for all 30 plates.
11. Centrifuge cell suspensions at 500*g* for 15 min and remove supernatant.
12. Re-suspend the cell pellet in 0.5 mL TD buffer per plate harvested (can be safely stored at –20°C).
13. Freeze cell pellets on dry ice, then thaw pellets (37°C) and vortex cell lysate. Repeat three times.
14. Add 100 U of Benzonase per mL of cell lysate and incubate for 45 min at 37°C.
15. Transfer lysate to 50 mL conical tube and centrifuge at 340*g* for 20 min.
16. Collect supernatant and add 0.55 g of CsCl per mL of supernatant and mix until dissolved. Adjust refractive index (RI) of supernatant to 1.372 with refractometer. If the RI

is too high, add small amounts of TD buffer. If RI is too low, add small amounts of CsCl.

17. Add supernatant to one Beckman Ultra-Clear Tube and fill tube to the very top with TD buffer adjusted with CsCl to a RI of 1.372.
18. Centrifuge tubes for 48–65 h at $\sim 182,300g_{ave}$ using low acceleration and low deceleration settings.
19. With a butterfly needle, puncture tube 2.5 cm above the bottom of the tube. Be sure to twist needle while pushing so that the plastic plug from the tube wall does not get stuck in the needle preventing the fractions from going through.
20. Using a clamp to control the flow, collect $\sim 500 \mu\text{L}$ fractions of purified virus in several 1.5 mL microcentrifuge tubes. Before collecting the next fraction, measure the RI of the previous fraction, keeping only those fractions with a RI between 1.375 and 1.367. These fractions typically contain the rAAV2 vector. The fraction in which the vector is found depends on the size of the transgene (i.e., larger transgene would mean fraction with a higher RI).
21. Combine fractions that have the same RI.
22. Perform QPCR on all fractions to measure vector titer with the ABI prism 7700 Sequence Detector as described in **Section 2.2**, step 10, but using TaqMan assays. The sequences for the forward primer, reverse primer, and probe are based on the Rous sarcoma virus promoter. The internal fluorogenic probe is labeled with the 6-FAM reporter dye (PE Applied Biosystems). The sequences used are as follows: forward – 5'-TGGATTGGACGAACCACTGA-3'; reverse – 5'-TCAAATGGCGTTTATTGTATCGA-3'; TaqMan probe – 5'-TTCCGCATTGCAGAGATAA-TTGTATTAAAGTGCCTA-3'. The reaction mixture is incubated at 50°C for 2 min (stage 1), 95°C for 10 min (stage 2), then denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min, repeated 40 times (stage 3). After completion, store selected fractions at 4°C.
23. Before using in vivo dialyze fractions against saline:
 - a. Put fraction in dialysis cassette.
 - b. Float cassette in 1 L of saline for 30 min while stirring at 4°C.
 - c. Change saline and repeat twice (total of 3 L of saline).
1. Weigh animal.
2. Inject 1 $\mu\text{L/g}$ ketamine:xylazine (3:2) intramuscularly into animal's hind leg – the animal is usually anesthetized within 5 min.

3.3. Methods for Delivery of Vector to Submandibular Glands

3. Place animal on specially constructed rack (**Fig. 1.4**):
 - a. Place upper jaw on wire – teeth over wire.
 - b. Pull lower jaw down with rubber band on rack.
 - c. Expand cheeks with wire spring.
 - d. Using a ball of cotton (~0.8 cm) to position (push) tongue back toward the throat.
4. Adjust binocular dissecting microscope to focus on both submandibular gland duct orifices, which are located slightly lateral to the midline of the floor of the mouth, about 4–5 mm posterior from the lower incisors. You should see two whitish papillae. The duct orifice is located on the ventral aspect, about half way down the papillae.
5. Pick up preformed cannula made from PE10 tubing for insertion. Using the scalpel blade, cut a bevel (~45°) on the narrow end in the middle of the thinnest part of the tubing. Note that younger animals tend to require thinner tubing, while older animals require thicker tubing.
6. Using very delicate forceps, pick up the beveled (narrow) end of the tubing (cannula), approximately 2 mm from the end of the tubing, and push it gently into the duct orifice. The angle of the cannula should be 45° to the floor of the mouth. Push the cannula approximately 3–4 mm into the duct. Be sure that the cannula is well sealed with the duct orifice by visualizing the fitness of the orifice rim to the cannula.
7. After successful cannula insertion, place a drop of Krazy glue at the site of insertion to secure the cannula.
8. Inject 0.5 mg/kg atropine intramuscularly, wait 10 min. While waiting, fill a syringe with sample or control solution for delivery. For rats the optimal volume to infuse is 200 µL, for mice 50 µL. Dilute vector in saline to the desired concentration.
9. Place distal end of cannula around the needle of a 0.3 cc syringe and inject sample slowly through the cannula into the gland.
10. Wait 10 min before gently removing the syringe to prevent backflow.
11. Gently remove cannulae and Krazy glue by pulling on tubing.

3.4. Methods for Rodent Saliva Collections

1. Pre-weigh 1.5 mL Eppendorf tube (one/mouse) and record.
2. Anesthetize mouse with ketamine (60 mg/kg) + xylazine (8 mg/kg).

3. Inject pilocarpine ($0.25 \mu\text{g/g}$) subcutaneously in back of neck. Pilocarpine will stimulate the salivary glands to secrete saliva. Normally, the saliva will start to flow about 3–5 min after pilocarpine is injected.
4. Place the mouse on a box about 8 cm high, with the head of the mouse over the edge of the box (**Fig. 1.2**).
5. Put one end of a Microhematocrit capillary tube under the mouse's tongue and the other end into the pre-weighed Eppendorf tube. Check capillary tube position and saliva flow constantly.
6. Monitor mouse carefully for signs of difficulty breathing and supply with oxygen if necessary.
7. Stop saliva collection after 20 min, making sure that all of the saliva drains from the mouse's mouth and capillary tube into the Eppendorf tube.
8. Weigh the tube, then subtract the pre-weighed value of the tube. The amount of saliva will be based on a specific gravity of 1 (i.e., 1 g/mL) and typically will be expressed as $\mu\text{L}/20 \text{ min}$. You can also convert the saliva output to $\mu\text{L/g}$ body weight or mg-gland weight .
9. The saliva can be directly used as output (end point measure) or be used to assess transgenic protein production or activity, if appropriate. Additionally, salivary composition can be determined, e.g., calcium, sodium, amylase, total protein, etc. Saliva should be stored at -80°C until assayed.

4. Notes

1. Making a cannula:
 - a. Cut a segment of tubing about 5 cm long.
 - b. Hold both ends of tubing between index finger and thumb (keep tubing a little bit loose).
 - c. Hold tubing about 15 cm above a delicate flame for a very short time – when the tubing begins to melt – remove from the flame and pull gently at both ends (not too much!).
 - d. Wait approximately 20 s for tubing to cool and solidify, store at room temperature.
2. Difficulty with preparation of the pJM17 plasmid (*see* gel picture of high-quality pJM17 preparation in **Fig. 1.1**). The pJM17 plasmid is a large plasmid, 40.07 kb. When preparing this plasmid it is necessary to be careful about two things.

First, remember when making this plasmid it results in a low yield. It is best to use 100 µg/mL of ampicillin for cultures of bacteria containing pJM17 and to culture the bacteria for less than 16 h. Second, during the plasmid extraction procedure, because of its size, this plasmid breaks easily. One needs to be very careful at each step to prevent unnecessary shearing of the plasmid. A high-quality preparation of pJM17 will show eight bands following 1% agarose gel electrophoresis after *Hind*III digestion (**Fig. 1.1**).

3. Test for viral activity of produced rAd5 and rAAV2
Seed a 12-well dish of 293 cells. At 90% confluency, transduce individual wells with equal amounts of viral particles (as determined by QPCR) from fractions being tested from the CsCl gradients. For rAd5 vectors use 10–100 viral particles/cell and for rAAV2 vectors use 100–1,000 viral particles/cell. Perform an ELISA or Western immunoblot on media or cell extracts later, as appropriate, from each transduced well to determine the viral fractions that best express transgenic protein and use those fractions for in vivo experiments.

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References

1. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C., and Thun, M. (2006) Cancer statistics. *CA Cancer J. Clin.* **56**, 106–130.
2. Mitsias, D. I., Kapsogeorgou, E. K., and Moutsopoulos, H. M. (2006) Sjögren's syndrome: why autoimmune epithelitis. *Oral Dis.* **12**, 523–532.
3. Kok, M. R., Yamano, S., Lodde, B. M., Wang, J., Couwenhoven, R. I., Yakar, S., Voutetakis, A., Leroith, D., Schmidt, M., Afione, S., Pillemer, S. R., Tsutsui, M. T., Tak, P. P., Chiorini, J. A., and Baum, B. J. (2003) Local adeno-associated virus-mediated interleukin 10 gene transfer has disease-modifying effects in a murine model of Sjögren's syndrome. *Hum. Gene Ther.* **14**, 1605–1618.
4. Baum, B. J. (1993) Principles of saliva secretion. *Ann. NY Acad. Sci.* **694**, 17–23.
5. Vitolo, J. M., and Baum, B. J. (2002) The use of gene transfer for the protection and repair of salivary glands. *Oral Dis.* **8**, 183–191.
6. Preston, G. M., and Agre, P. (1991) Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. *Proc. Natl. Acad. Sci. USA.* **88**, 11110–11114.
7. Baum, B. J., Wellner, R. B., and Zheng, C. (2002) Gene transfer to salivary glands. *Int. Rev. Cytol.* **213**, 93–146.

8. Mastrangeli, A., O'Connell, B., Aladib, W., Fox, P. C., Baum, B. J., and Crystal, R. G. (1994) Direct in vivo adenovirus-mediated gene transfer to salivary glands. *Am. J. Physiol.* **266**, G1146–G1155.
9. McGrory, W. J., Bautista, D. S., and Graham, F. L. (1988) A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology*. **163**, 614–617.
10. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**, 59–74.
11. Chiorini, J. A., Wendtner, C. M., Urcelay, E., Safer, B., Hallek, M., and Kotin, R. M. (1995) High-efficiency transfer of the T cell co-stimulatory molecule B7-2 to lymphoid cells using high-titer recombinant adeno-associated virus vectors. *Hum. Gene Ther.* **6**, 1531–1541.
12. Vitolo, J. M., Cotrim, A. P., Sowers, A. L., Russo, A., Wellner, R. B., Pillemer, S. R., Mitchell, J. B., and Baum, B. J. (2004) The stable nitroxide Tempol facilitates salivary gland protection during head and neck irradiation in a mouse model. *Clin. Cancer Res.* **10**, 1807–1812.
13. Cotrim, A. P., Hyodo, F., Matsumoto, K., Sowers, A. L., Cook, J. A., Baum, B. J., Krishna, M. C., and Mitchell, J. B. (2007) Differential radiation protection of salivary glands versus tumor by Tempol with accompanying tissue assessment of Tempol by magnetic resonance imaging. *Clin. Cancer Res.* **13**, 4928–4933.
14. Grimm, D., Kern, A., Rittner, K., and Kleinschmidt, J. A. (1998) Novel tools for production and purification of recombinant adeno-associated virus vectors. *Hum. Gene Ther.* **9**, 2745–2760.

Chapter 2

Collection, Storage, and Processing of Saliva Samples for Downstream Molecular Applications

Bradley Stephen Henson and David T. Wong

Abstract

Saliva is an ideal translational research tool and diagnostic medium and is being used in novel ways to provide molecular biomarkers for a variety of oral and systemic diseases and conditions. The ability to analyze saliva to monitor health and disease is a highly desirable goal for oral health promotion and research. Saliva has been used to detect caries risk, periodontitis, oral cancer, breast cancer, salivary gland diseases, and systemic disorders such as hepatitis, HIV and HCV. Technology advancement has allowed high-throughput studies to be performed at a scale unrealized previously and is serving to advance the discovery and validation of salivary disease biomarkers. Of course, successful measurement of salivary analytes requires optimal collection, processing, and storage procedures and conditions. This chapter describes protocols for saliva collection, processing, and storage for the molecular analysis of salivary diagnostic constituents.

Key words: Saliva, saliva collection, saliva processing, diagnostics, submandibular, parotid, sublingual.

1. Introduction

Saliva is a mirror of the body, reflecting virtually the entire spectrum of normal and disease states and its use as a diagnostic fluid meets the demands for an inexpensive, non-invasive, and accessible diagnostic tool. Discovery of analytes in the saliva of normal and diseased subjects suggests a very exciting function of saliva; a local and systemic diagnostic tool. The ability to analyze saliva to monitor health and disease is a highly desirable goal for oral health promotion and research. Saliva has been used to detect

caries risk (1–3), periodontitis (4), oral cancer (5), breast cancer (6–11), salivary gland diseases (12), and systemic disorders such as hepatitis, HIV, and HCV (13–17). For example, rapid point-of-care HIV tests utilize saliva, gingival crevicular fluid, or oral mucosal fluid to rapidly provide test results to patients (17, 18). The ease of collecting, handling, and testing saliva has led to its use for determining hormone levels, including estradiol, progesterone and testosterone, DHEA, and cortisol (19). This is particularly important in the case of estradiol, as it can be an indicator of premature birth and low birth weight babies (20). Numerous drugs are detectable in oral fluid and can even be quantified in saliva as a viable substitute to testing in blood, and as a result, salivary diagnostic technology is currently utilized to test for drugs of abuse, such as cocaine (21, 22), methamphetamines (23, 24), and opiates (25). Additionally, saliva can also be used for therapeutic monitoring of drugs, such as digoxin (26, 27), methadone (28), and some anticonvulsants (29).

However, the development of disease biomarkers has not been fully realized due to an overall low concentration of these markers in saliva when compared to serum. Today, highly sensitive and high-throughput assays such as microarray, mass spectrometry, reverse transcriptase polymerase chain reaction (RT-PCR), and nano-scale sensors can measure proteins and RNAs even at low concentrations in saliva, thus expanding the utility of saliva as a diagnostic fluid.

Technology advancement has allowed high-throughput studies to be performed at a scale unrealized previously. Additionally, RNA detection using microarrays and protein analysis using mass spectrometry has allowed researchers to monitor analyte expression changes from a single biological sample. The result has been a resurgence in saliva-based diagnostic research. In 2004, a multi-institutional, multidisciplinary research consortium was initiated and funded by the National Institute of Dental and Craniofacial Research (NIDCR) to generate a complete catalogue of all salivary secretory proteins (Human Salivary Proteome Project, <http://www.skb.ucla.edu>). This collaborative endeavor yielded 1,166 identifications in ductal fluid: 914 in parotid and 917 in submandibular/sublingual saliva (30). Similarly, RNA has been found to exist in saliva (5) and this discovery has spurred the development of saliva transcriptome analyses. These partially degraded RNAs can be detected by microarrays and a group of “core” RNA species have been consistently detected in whole and supernatant saliva samples (5). In the laboratories of the authors, micro-channel electrophoresis has been performed using the Agilent Bioanalyzer (Agilent Technologies) in combination with the Agilent Pico Chip in order to analyze the presence of RNA in saliva. To validate that the signal seen is representative of RNA in addition to DNA, RNase and DNase treatments were performed.

RNA from whole and supernatant saliva is completely degraded by RNase treatment. The treatment with DNase reduces the area of the observed peak by approximately 70%, demonstrating that RNA is genuinely present in human saliva.

A very important development in oral disease diagnosis has been the identification of salivary markers for the detection of oral cancer. Based on expression or deletion of specific molecular markers, molecular staging profiles have been identified in saliva that act as objective prognostic indicators. Recently, several mRNA biomarkers of oral squamous cell carcinoma (OSCC) were identified (5). Unstimulated saliva was collected from patients ($n = 32$) with primary OSCC as well as normal subjects ($n = 32$) with matched age, gender, and smoking history. RNA was then isolated from saliva supernatant and amplified using T7 RNA polymerase. The product was then placed on Human Genome U133A microarrays and gene expression patterns were analyzed on 10 OSCC samples and matched controls. Analysis yielded seven cancer-related mRNA biomarkers that exhibited at least a 3.5-fold elevation in OSCC saliva ($P < 0.01$): *DUSP1*, *HA3*, *OAZ1*, *SI00P*, *SAT*, *IL8*, and *IL1B*. These genes were validated using quantitative polymerase chain reaction (qPCR). Then, the predictive power of these salivary mRNA biomarkers was analyzed by receiver operating characteristic curve and classification models. When the combination of these biomarkers is used for OSCC detection, a sensitivity of 91% and specificity of 91% was observed when compared to controls.

Similarly, Hu et al. (31) recently demonstrated the presence of informative protein biomarkers in the human saliva proteome for detection of OSCC. In this study, shotgun proteomics based on C4 reversed-phase liquid chromatography (RP-LC) for protein pre-fractionation, capillary RP-LC with quadruple time-of-flight mass spectrometry, and Mascot sequence database searching as well as two-dimensional gel electrophoresis (2DE) analysis were used to profile proteins in whole saliva samples from oropharyngeal cancer patients and healthy controls. Immunoassay verification was then performed on a separate cohort of subjects. As a result, five candidate biomarkers for OSCC were verified: Mac-2 binding protein (M2BP), calgranulin B (MRP14), protectin (CD59), profilin, and catalase (31). The combination of these candidate biomarkers yielded sensitivity and specificity values of 90 and 83%, respectively (31).

Of course, successful measurement of salivary analytes requires optimal collection, processing, and storage procedures and conditions. The materials and techniques used to collect samples may influence the accuracy of testing. It is important to remain consistent in collection procedures both across all patients and within all collection visits for each individual. For example, the initial 2 min of parotid secretion should be discarded

prior to the collection because this period is thought to be one of gland accommodation where salivary constituents are variable (32, 33). It is also important to determine the appropriate collection materials for the targeted analyte. For example, steroid hormones, such as cortisol, usually necessitate the use of low-affinity plastic containers to prevent protein binding to the walls of the tube (34). Saliva collection protocols have been thoroughly established for both whole saliva collection and glandular secretion collection (32, 35–38) and as the field of salivary diagnostics expands in scope, so too will the utility of these procedures. This chapter describes protocols for saliva collection, processing, and storage for the molecular analysis of salivary diagnostic constituents.

2. Materials

2.1. Saliva Collection Procedures

2.1.1. Whole Saliva Collection

- (1) 50 mL sterile tube and paper/styrofoam cup
- (2) Crushed ice and container
- (3) Distilled water

2.1.2. Ductal Secretion Collection

- (1) Sterile-modified Carlson–Crittenden/Lashley cup (35, 39, 40) fitted with appropriate polyvinyl chlorate tubing. This device requires suction, which can be provided by a dental unit suction, laboratory suction bulb, or oil-free portable vacuum pump

2.1.2.1. Parotid Secretions

- (2) Low-affinity conical plastic collection tubes on ice
- (3) Approximately 5 mL of sterile 2% w/v aqueous citric acid solution, store at room temperature (RT)
- (4) Cotton tip applicators

2.1.2.2. Submandibular and Sublingual Secretions

- (1) Submandibular and sublingual saliva collector described by Wolfe et al. (36), fitted with a sterile 100 μ L pipette tip and a low-affinity plastic conical collection tube. This device requires suction, which can be provided by a dental unit suction or oil-free portable vacuum pump
- (2) Distilled water
- (3) Sterile cotton sponges, dental mirror, and forceps

- (4) Approximately 5 mL of sterile 2% w/v aqueous citric acid solution
- (5) Sterile cotton tip applicators

2.2. Processing and Storage

All procedures must be performed on ice

Equipment and supplies:

- (1) Laboratory vortex mixer
- (2) Refrigerated centrifuge able to accommodate 50 mL tubes
- (3) Cryotubes able to accommodate -80°C temperatures
- (4) -80°C freezer

Reagents:

- (1) Aprotinin from standard commercial stock solution stored at 4°C
- (2) 400 mM Na_3OV_4 standard stock: 147.12 mg/2 mL H_2O . Adjust the pH to 10.0 and boil for approximately 10 min until the solution remains colorless. Store at room temperature (RT)
- (3) 10 mg/mL phenylmethylsulfonyl fluoride (PMSF) standard: 100 mg PMSF dissolved in 10 mL isopropanol, mixed by gentle inversion. Store at RT
- (4) SUPERase Inhibitor (Ambion) stored at -20°C

3. Methods

3.1. Saliva Collection Procedures

3.1.1. Whole Saliva Collection

- (1) Tell the subject what time you plan to collect saliva (please aim for 8–10 a.m. if possible) and ask the subject to refrain from eating, drinking, or oral hygiene procedures for at least 1 h prior to the collection.
- (2) Give the subject distilled drinking water and ask that they rinse their mouth out well for 1 min. The subject can then expectorate or swallow the water.
- (3) Five minutes after this oral rinse, ask the subject to spit into a 50 mL sterile tube. Encourage the subjects to place the tube on ice while collecting more saliva (*see Note 1*).
- (4) Collect approximately 5 mL volume of saliva.
- (5) Return to the laboratory immediately for processing. Processing should occur within a 1 h window of time (*see Note 2*).

3.1.2. Ductal Secretion Collection

3.1.2.1. Parotid Secretions

- (1) Affix a modified Carlson–Crittenden cup (35, 39, 40) over the orifice of the Stenson's duct.
- (2) Collect unstimulated saliva for approximately 15–20 min into a conical plastic tube on ice.
- (3) Collect in a stimulated fashion by the repeated application of an aqueous citric acid solution (approximately 2% w/v) to the dorsal surface of the tongue (*see* **Note 3**).

3.1.2.2. Submandibular and Sublingual Secretions

- (1) Separate submandibular and sublingual secretions should be harvested using a saliva collector described by Wolfe et al. (36), which can be fitted with a sterile 100 μL pipette tip and a plastic conical collection tube.
- (2) Ask subjects to rinse their mouths with distilled water for 1 min followed by expectoration.
- (3) Place sterile cotton sponges in the floor of the mouth and over the buccal mucosal areas to occlude the parotid and sublingual ducts.
- (4) Collect acid-stimulated saliva by placing the micropipette tip of the collection device, under light suction, at the orifice of the Wharton's duct.
- (5) Collect sublingual saliva samples in a similar fashion by placing the tip of the saliva collection device over the sublingual ducts while covering the submandibular and parotid ducts with sterile cotton (*see* **Note 4**).

3.2. Processing and Storage

All procedures must be performed on ice.

- (1) Divide the saliva sample into multiple 330 μL samples placed in cryotubes able to accommodate -80°C temperatures.
- (2) Each 330 μL sample can now be further processed for storage according to the anticipated endpoint analysis, i.e., protein or RNA analysis.
 - (a) For samples intended for protein analysis add the following protease inhibitors to each 330 μL volume of saliva:
 - i. 0.33 μL aprotinin. Invert gently to mix.
 - ii. 1 μL Na_3OV_4 (from standard stock of 400 mM). Invert gently to mix.
 - iii. 3.3 μL PMSF (standard stock of 10 mg/mL). Invert gently to mix.
 - (b) For samples intended for RNA analysis add the following RNase inhibitors to each 330 μL volume of saliva (*see* **Note 5**):

- i. 1.65 μL (5 μL SI/1 mL sample) SUPERase Inhibitor (Ambion).

(3) Store all fractions at -80°C .

For supernatant samples:

Some salivary diagnostic protocols may require supernatant saliva drawn from whole saliva fractions.

- (1) Briefly vortex the whole saliva sample (fewer than 20 s) so that it whirls up the sides of the tube.
- (2) Spin the entire sample at 2,600g for 15 min at 4°C (*see Note 6*).
- (3) Remove the supernatant taking care not to disturb the “pellet” at the bottom of the tube and transfer the fractions to appropriately labeled cryotubes. Similarly, transfer the resultant pellet to a fresh tube.
- (4) Add the RNase inhibitor and protease inhibitors to the supernatant fractions as described above. Note: Do not add these reagents to the pellet.
- (5) Store all fractions and the pellet at -80°C .

4. Notes

1. Remind the subjects not to cough up mucus as the goal is to passively collect saliva, not phlegm.
2. It is imperative that all saliva sample processing occurs as soon after collection as possible and that all samples remain on ice at all times. Prior to actually collecting the saliva sample from the patient, set the centrifuge to 4°C to make sure that it has reached the proper temperature for processing. Also, chill any tube holders and cryotubes in the freezer or refrigerator prior to processing (-20°C or -80°C).
3. If citric acid application to the dorsal surface of the tongue does not stimulate saliva flow, swab the lateral surfaces of the tongue every 30 s.
4. Due to its inherent viscosity, it may be difficult to collect sublingual saliva through the orifice of the narrow micropipette tip, and it may be necessary to widen the orifice by clipping off approximately 1 mm from the collection end with sterile scissors.
5. One should try to reduce freeze/thaw cycles of all samples to prevent nucleic acid and protein degradation. Additionally, exposing a sample to the air can result in oxidation and inactivation of dithiothreitol (DTT), a reducing reagent

traditionally present in RNase inhibitor solutions. It is important to note that SUPERase Inhibitor has no DTT requirement, but is still fully functional in DTT containing preparations (adapted from Ambion product information documentation).

6. Following the supernatant centrifugation it may be observed that a discernable pellet has not formed at the bottom of the tube. It is often necessary to re-vortex the sample for approximately 15 s and repeat the centrifugation step.

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References

1. Bradshaw, D. J., and Marsh, P. D. (1998) Analysis of pH-driven disruption of oral microbial communities in vitro. *Caries Res.* **32**, 456–462.
2. Bratthall, D., and Hansel Petersson, G. (2005) Cariogram – a multifactorial risk assessment model for a multifactorial disease. *Community Dent. Oral Epidemiol.* **33**, 256–264.
3. Larmas, M. (1992) Saliva and dental caries: diagnostic tests for normal dental practice. *Int. Dent. J.* **42**, 199–208.
4. Christodoulides, N., Floriano, P. N., Miller, C. S., Ebersole, J. L., Mohanty, S., Dharshan, P., Griffin, M., Lennart, A., Ballard, K. L., King, C. P., Jr., Langub, M. C., Kryscio, R. J., Thomas, M. V., and McDevitt, J. T. (2007) Lab-on-a-chip methods for point-of-care measurements of salivary biomarkers of periodontitis. *Ann. NY Acad. Sci.* **1098**, 411–428.
5. Li, Y., St John, M. A., Zhou, X., Kim, Y., Sinha, U., Jordan, R. C., Eisele, D., Abemayor, E., Elashoff, D., Park, N. H., and Wong, D. T. (2004) Salivary transcriptome diagnostics for oral cancer detection. *Clin. Cancer Res.* **10**, 8442–8450.
6. Streckfus, C., and Bigler, L. (2005) The use of soluble, salivary c-erbB-2 for the detection and post-operative follow-up of breast cancer in women: the results of a five-year translational research study. *Adv. Dent. Res.* **18**, 17–24.
7. Streckfus, C., Bigler, L., Dellinger, T., Dai, X., Cox, W. J., McArthur, A., Kingman, A., and Thigpen, J. T. (2001) Reliability assessment of soluble c-erbB-2 concentrations in the saliva of healthy women and men. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **91**, 174–179.
8. Streckfus, C., Bigler, L., Dellinger, T., Dai, X., Kingman, A., and Thigpen, J. T. (2000) The presence of soluble c-erbB-2 in saliva and serum among women with breast carcinoma: a preliminary study. *Clin. Cancer Res.* **6**, 2363–2370.
9. Streckfus, C., Bigler, L., Dellinger, T., Pfeifer, M., Rose, A., and Thigpen, J. T. (1999) CA 15-3 and c-erbB-2 presence in the saliva of women. *Clin. Oral Invest.* **3**, 138–143.
10. Streckfus, C., Bigler, L., Tucci, M., and Thigpen, J. T. (2000) A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. *Cancer Invest.* **18**, 101–109.
11. Streckfus, C. F., Bigler, L., Dellinger, T., Kuhn, M., Chouinard, N., and Dai, X. (2004) The expression of the c-erbB-2 receptor protein in glandular salivary secretions. *J. Oral Pathol. Med.* **33**, 595–600.
12. Hu, S., Zhou, M., Jiang, J., Wang, J., Elashoff, D., Gorra, S., Michie, S. A., Spijkervet, F. K., Bootsma, H., Kallenberg, C. G., Vissink, A., Horvath, S., and Wong, D. T. (2009) Systems biology analysis of sjogren's

- syndrome and mucosa-associated lymphoid tissue lymphoma in parotid glands. *Arthritis Rheum.* **60**, 81–92.
13. Elsansa, S., Sikuler, E., Yaari, A., Shemer-Avni, Y., Abu-Shakra, M., Buskila, D., Katzman, P., Naggan, L., and Margalith, M. (1998) HCV antibodies in saliva and urine. *J. Med. Virol.* **55**, 24–27.
 14. Yaari, A., Tovbin, D., Zlotnick, M., Mostoslavsky, M., Shemer-Avni, Y., Hanuka, N., Burbea, Z., Katzir, Z., Storch, S., and Margalith, M. (2006) Detection of HCV salivary antibodies by a simple and rapid test. *J. Virol. Methods.* **133**, 1–5.
 15. Mortimer, P. P., and Parry, J. V. (1994) Detection of antibody to HIV in saliva: a brief review. *Clin. Diagn. Virol.* **2**, 231–243.
 16. Tamashiro, H., and Constantine, N. T. (1994) Serological diagnosis of HIV infection using oral fluid samples. *Bull. World Health Organ.* **72**, 135–143.
 17. Hodinka, R. L., Nagashunmugam, T., and Malamud, D. (1998) Detection of human immunodeficiency virus antibodies in oral fluids. *Clin. Diagn. Lab. Immunol.* **5**, 419–426.
 18. Fernandez Rodriguez, E., Carcaba Fernandez, V., Rodriguez Junquera, M., Alfonso Megido, J., Garcia Amorin, Z., and Garcia Alonso, S. (1994) Detection of HIV antibodies in saliva using a rapid diagnostic immunoenzyme assay. *Rev. Clin. Esp.* **194**, 523–525.
 19. Groschl, M. (2008) Current status of salivary hormone analysis. *Clin. Chem.* **54**, 1759–1769.
 20. Lawrence, H. P. (2002) Salivary markers of systemic disease: noninvasive diagnosis of disease and monitoring of general health. *J. Can. Dent. Assoc.* **68**, 170–174.
 21. Kato, K., Hills Grove, M., Weinhold, L., Gorelick, D. A., Darwin, W. D., and Cone, E. J. (1993) Cocaine and metabolite excretion in saliva under stimulated and nonstimulated conditions. *J. Anal. Toxicol.* **17**, 338–341.
 22. Schramm, W., Craig, P. A., Smith, R. H., and Berger, G. E. (1993) Cocaine and benzoylecgonine in saliva, serum, and urine. *Clin. Chem.* **39**, 481–487.
 23. Huestis, M. A., and Cone, E. J. (2004) Relationship of Delta 9-tetrahydrocannabinol concentrations in oral fluid and plasma after controlled administration of smoked cannabis. *J. Anal. Toxicol.* **28**, 394–399.
 24. Schepers, R. J., Oyler, J. M., Joseph, R. E., Jr., Cone, E. J., Moolchan, E. T., and Huestis, M. A. (2003) Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. *Clin. Chem.* **49**, 121–132.
 25. Cone, E. J. (1990) Testing human hair for drugs of abuse. I. Individual dose and time profiles of morphine and codeine in plasma, saliva, urine, and beard compared to drug-induced effects on pupils and behavior. *J. Anal. Toxicol.* **14**, 1–7.
 26. Cohen, A. F., and Mattie, H. (1979) Salivary electrolytes in the detection of digoxin intoxication. *Neth. J. Med.* **22**, 149–152.
 27. Swanson, M., Cacace, L., Chun, G., and Itano, M. (1973) Saliva calcium and potassium concentrations in the detection of digitalis toxicity. *Circulation.* **47**, 736–743.
 28. Chikhi-Chorfi, N., Pham-Huy, C., Galons, H., Manuel, N., Lowenstein, W., Warnet, J. M., and Claude, J. R. (1998) Rapid determination of methadone and its major metabolite in biological fluids by gas-liquid chromatography with thermionic detection for maintenance treatment of opiate addicts. *J. Chromatogr. B Biomed. Sci. Appl.* **718**, 278–284.
 29. Hartley, R., Luccock, M., Becker, M., Smith, I. J., and Forsythe, W. I. (1986) Solid-phase extraction of acetazolamide from biological fluids and subsequent analysis by high-performance liquid chromatography. *J. Chromatogr.* **377**, 295–305.
 30. Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G. S., Boonthueung, P., Cociorva, D., Delahunty, C. M., Denny, T., Dunsmore, J., Faull, K. F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S. C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J. A., Ogorzalek Loo, R. R., Malamud, D., Melvin, J. E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S. K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M. A., Takashima, J., Than, S., Wang, J., Whitelegge, J. P., Witkowska, H. E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D. T., Yates, J. R., 3rd, and Fisher, S. J. (2008) The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *J. Proteome. Res.* **7**, 1994–2006.
 31. Jiang, J., Park, N. J., Hu, S., and Wong, D. T. (2008) A universal pre-analytic solution for concurrent stabilization of salivary proteins, RNA and DNA at ambient temperature. *Arch. Oral Biol.* **54**, 268–273.
 32. Baum, B. J. (1981) Evaluation of stimulated parotid saliva flow rate in different age groups. *J. Dent. Res.* **60**, 1292–1296.

33. Dawes, C. (1969) The effects of flow rate and duration of stimulation on the concentrations of protein and the main electrolytes in human parotid saliva. *Arch. Oral Biol.* **14**, 277–294.
34. Hanrahan, K., McCarthy, A. M., Kleiber, C., Lutgendorf, S., and Tsalikian, E. (2006) Strategies for salivary cortisol collection and analysis in research with children. *Appl. Nurs. Res.* **19**, 95–101.
35. Carlson, A. V., and Crittenden, A. L. (1910) The relation of ptyalin concentration to the diet and to the rate of secretion of the saliva. *Am. J. Physiol.* **26**, 169–177.
36. Wolf, R. O. (1964) Regulated vacuum system for collecting submaxillary and sublingual saliva. *J. Dent. Res.* **43**, 303.
37. Tylanda, C. A., Ship, J. A., Fox, P. C., and Baum, B. J. (1988) Evaluation of submandibular salivary flow rate in different age groups. *J. Dent. Res.* **67**, 1225–1228.
38. Fox, P. C., van der Ven, P. F., Sonies, B. C., Weiffenbach, J. M., and Baum, B. J. (1985) Xerostomia: evaluation of a symptom with increasing significance. *J. Am. Dent. Assoc.* **110**, 519–525.
39. Heft, M. W., and Baum, B. J. (1984) Unstimulated and stimulated parotid salivary flow rate in individuals of different ages. *J. Dent. Res.* **63**, 1182–1185.
40. Lashley, K. S. (1916) Reflex secretion of the human parotid gland. *J. Exp. Psychol.* **1**, 461–493.

Chapter 3

Proteomic Analysis of Saliva: 2D Gel Electrophoresis, LC-MS/MS, and Western Blotting

Shen Hu, Jiang Jiang, and David T. Wong

Abstract

Saliva harbors a wide spectrum of proteins that may reflect the health/disease status in the human body. Profiling of the proteins in saliva from a disease population can potentially yield valuable clinical parameters to be used for diagnosis and prognosis of the disease. Advances in proteomic technologies have enabled comprehensive profiling of protein expression in cells, tissue, and body fluids. When applied to readily accessible saliva samples from disease patients for biomarker study, such a global approach allows attaining the most discriminatory protein biomarkers that can best predict the disease status. In this chapter, we describe the protocols for proteomic analysis of saliva using 2D gel electrophoresis, Western blotting, and LC-MS/MS.

Key words: Salivary proteomics, salivary diagnostics, tandem mass spectrometry, 2D gel electrophoresis.

1. Introduction

Saliva has been increasingly recognized as an acceptable alternative to blood for use in diagnostic tests because salivary testing is safe, low cost, and non-invasive (1, 2). Due to easy sample collection and processing, saliva represents a readily accessible body fluid that may be repeatedly sampled for long-term monitoring of disease progression or in vivo assessment of efficacy and toxicity of drug treatment.

Human saliva contains a large number of proteins, which play important roles in maintaining oral/general health and may serve as biomarkers to survey disease status. An in-depth analysis

of the human saliva proteome as well as their posttranslational modifications can therefore provide a valuable resource for oral biology and saliva diagnostics research (3–13). To date, a total of 1,939 unique proteins have been compiled from 19,474 unique peptide sequences identified from whole and ductal saliva samples, including 740 proteins from both whole and ductal saliva. Shotgun proteomics based on multidimensional chromatography with tandem mass spectrometry (MS/MS) represent a major technology used for comprehensive identification of saliva proteins. Among the 1,939 proteins identified from human saliva, a total of 597 are also present in the human plasma proteome (14).

MS-based proteomic approaches have been used to identify saliva biomarkers for Sjögren's syndrome (SS), which is a systemic autoimmune disease characterized by dry eyes and dry mouth (15–17). It was found that the saliva proteomic profiles of SS patients are a mixture of increased inflammatory proteins and decreased acinar proteins as compared with those in non-SS controls. Promising biomarkers derived from our proteomic study included alpha-enolase, beta-microglobulin, cathepsin D, and carbonic anhydrase I, which have been successfully validated in a new group of SS, systemic lupus erythematosus (SLE, autoimmune disease control), and healthy control subjects (unpublished results). Diagnosing SS is complicated by the range of symptoms a patient may manifest, and the similarity between symptoms from SS and those caused by other autoimmune disorders such as SLE and rheumatoid arthritis. The availability of these unique saliva protein biomarkers may lead to a simple clinical tool for non-invasive and highly specific diagnosis of SS in the future. Meanwhile, saliva proteomics has been used in searching for biomarkers for oral and breast cancers (18, 19). By using subtractive proteomics followed by immunoassays for validation, we discovered five potential protein biomarkers (calgranulin B, Mac-2 binding protein, CD-59, catalase, and profilin) in saliva for oral cancer. The combination of these candidate biomarkers yielded a sensitivity of 90% and a specificity of 83% in detecting oral squamous cell carcinoma. Proteomics analysis of cells in whole saliva from oral cancer patients also provided an approach that may reveal protein biomarkers for oral cancer detection (20).

2. Materials

2.1. 2D Gel Electrophoresis of Saliva Proteins

1. SYPRO Ruby protein stain (Invitrogen)
2. 2D-Quant total protein assay kit (Amersham)
3. Agarose sealing solution: 25 mM Tris base, 192 mM glycine, 0.1% SDS, 0.5% agarose, 0.002% bromophenol

4. Rehydration buffer: 7 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS, 5% glycerol, 10% isopropanol
5. Equilibration buffer: 6 M urea, 0.375 M Tris-HCl, 2% SDS, 20% glycerol, pH 8.8
6. 2% DTT in equilibration buffer
7. 2.5% iodoacetic acid in equilibration buffer
8. TGS running buffer (Bio-Rad)
9. ReadyStrip[®] pH 3–10 NL IPG strips (Bio-Rad)
10. MultiMark[®] Multi-Colored Standard (Invitrogen)
11. Protean II ready gel 8–16% Tris-HCl (Bio-Rad)
12. Protean IEF cell (Bio-Rad)
13. FX scanner (Bio-Rad)
14. ProteomeWorks spot cutter (Bio-Rad)

2.2. Protein Identification

1. Ammonium bicarbonate (Fisher Scientific)
2. Acetonitrile (ACN; Sigma-Aldrich)
3. 10 mM DTT (Bio-Rad) in 100 mM ammonium bicarbonate
4. 50 mM iodoacetic acid (Sigma-Aldrich) in 100 mM ammonium bicarbonate
5. Trifluoric acid (TFA, Sigma-Aldrich)
6. Formic acid (Sigma-Aldrich)
7. Sequencing-grade trypsin (Promega)
8. Nano-LC system (Eksigent Technology)
9. Linear ion trap MS system (LTQ XL, Thermo-Fisher Scientific)
10. PicoTip emitter (New Objective)
11. LC mobile phase A: 95% H₂O/5% ACN/0.1% formic acid
12. LC mobile phase B: 95% ACN/0.1% formic acid

2.3. Western Blot Analysis of Saliva Proteins

1. NuPAGE Bis-Tris pre-cast minigel (Invitrogen)
2. MES SDS running buffer (Invitrogen)
3. TBS(10×): 100 mM Tris base, 1.5 M NaCl, pH 7.6
4. TBST(1×): 200 mL TBS(10×), 20 mL 10% Tween 20, 1,780 mL H₂O
5. 5% nonfat dry milk (NFDM) in freshly prepared TBST(1×)
6. See Blue Plus 2[®] pre-stained protein standards (Invitrogen)
7. ECL detection kit (Amersham)
8. HyBlot CL autoradiography film (Denville Scientific)
9. iBlot dry blot system and nitrocellulose transfer stack kit (Invitrogen)

2.4. Shotgun Proteomics

1. Ammonium bicarbonate (Fisher Scientific)
2. Acetonitrile (Sigma-Aldrich)
3. DTT (Bio-Rad)
4. Iodoacetic acid (Sigma-Aldrich)
5. Trifluoroic acid (TFA, Sigma-Aldrich)
6. Formic acid (Sigma-Aldrich)
7. Sequencing-grade trypsin (Promega)
8. Micro-scale HPLC (HP1100, Agilent Technologies)
9. C4 reversed-phase LC column (Vydac, particle size, 5 μm ; 250 $\times$ 2.1 mm I.D.)
10. Nano-LC system (Dionex)
11. QqTOF mass spectrometer (QSTAR XL, Applied Biosystems)
12. PicoTip emitter (New Objective)
13. LC Packings PepMap C18 column (75 μm $\times$ 150 mm; particle size, 5 μm)
14. LC mobile phase A for pre-fractionation: 0.1% formic acid
15. LC mobile phase B for pre-fractionation: ACN/0.1% formic acid
16. LC mobile phase A for LC-QqTOF MS: 95% H_2O /5% ACN/0.1% formic acid
17. LC mobile phase B for LC-QqTOF MS: 95% ACN/0.1% formic acid

3. Methods

A carefully designed sample collection/pretreatment protocol is crucial to the success of a saliva proteomics project (*see* **Notes 1** and **2**). The proteins in saliva can be profiled first by 2D gel electrophoresis or liquid chromatography, followed by tryptic digestion, LC-MS/MS, and database search to identify the selected protein targets of interest. Western blotting can be used to quantify protein levels for validation purpose.

3.1. 2D Gel Electrophoresis of Saliva Proteins

3.1.1. Total Protein Assay

Total protein concentration of each sample was determined with 2D-Quant protein kit. 2D gel analysis can be performed on

individual whole saliva samples or pooled samples (e.g., pooled cancer or control samples) prepared with equal contribution of proteins from each individual sample.

3.1.2. Protein Precipitation

1. In order to precipitate proteins, add 9× volume of absolute ethanol (pre-chilled at -20°C) to a saliva sample, mix briefly, and then store at -20°C for at least 2 h or preferably overnight.
2. Spin the sample down at 14,000*g* for 20 min (4°C). Remove the supernatant and collect the remaining pellet.

3.1.3. IEF – First Dimension

1. The pellet is then re-suspended in 300 μL (for 17 cm IPG strip) of rehydration buffer and 2 μL of ampholyte (pH 3–10NL) and vortexed for 30 s.
2. The re-suspended sample is then loaded into an IEF cell, and an IPG strip is layered on the top of the sample with gel side facing down.
3. After 45 min, cover the IPG strip with mineral oil to prevent evaporation and apply a low voltage (50 V) for active rehydration overnight.
4. Next day, discard the mineral oil and place the IPG strip in the IEF cell with gel side facing down. Insert wet filter paper wicks between electrodes and IPG strips to absorb salts during electrophoresis. Afterward, cover the IPG strips with mineral oil and apply IEF voltage program. For saliva protein samples, we use the following IEF program: 250 V (rapid), 5 h; 500 V (linear), 3 h; 500 V (rapid), 5 h; 3,000 V (linear), 4 h; 3,000 V (rapid), 4 h; 10,000 V (linear), 5 h; 10,000 V (rapid), 10 h; and 250 V (rapid), 99 h.

3.1.4. SDS-PAGE – Second Dimension

1. After IEF, the IPG strip is rinsed with fresh 2% DTT and then 2.5% iodoacetic acid solutions for 10 min each.
2. After briefly rinsing with the TGS running buffer, the strip is placed onto an 8–16% Tris–HCl Protean II ready gel.
3. A paper wick blotted with pre-stained MultiMark[®] Multi-Colored protein standards was inserted at the left side of the gel and agarose solution was then used to seal the IPG strip.
4. SDS-PAGE is performed under a separation voltage of 100–200 V.

3.1.5. Staining and Image Analysis

1. After fixing in 7% acetic acid–10% methanol for 30 min, stain the gel with the SYPRO Ruby protein stain in the dark for overnight.
2. Next morning, the gel is de-stained in 7% acetic acid–10% methanol in the dark for at least 2 h.

3. The gel images are then acquired with the Bio-Rad FX scanner and analyzed with the PDQuest program.
4. Gel spots of interest are excised using the ProteomeWorks spot cutter.

3.2. Identification of Proteins in 2D Gel Spots

3.2.1. In-Gel Tryptic Digestion

3.2.1.1. Wash Gel Spots

1. Gel slices are washed with 100 μL of 50 mM NH_4HCO_3 and 50% CH_3CN in a 1.5 mL micro-centrifuge tube. Wash for 10 min and then remove the supernatant.
2. 30 μL of 100% CH_3CN is added to the tube. The gel slices are washed again for 10 min and the supernatant is removed. Step 1–2 may be repeated if necessary.
3. Finally, the gel slices are dried in a Speedvac for about 5–10 min.

3.2.1.2. Reduce Disulfide Bonds and Block Free Cysteine Sulphydryl Bonds

1. To reduce the disulfide bonds of proteins, 20 μL of 10 mM DTT is added to gel spots and incubated at 60°C for 1 h. The supernatant is discarded.
2. 20 μL of 50 mM iodoacetic acid is added to the gel spots. The incubation should be performed in the dark at 45°C for about 45 min and finally the supernatant is discarded.

3.2.1.3. Wash Gel Spots

1. The gel spots are washed with 50 μL of 100 mM NH_4HCO_3 buffer for 10 min.
2. After the supernatant is removed, 50 μL of 100% CH_3CN is added for another 10-min washing and the supernatant is discarded.
3. Repeat steps 1 and 2 and dry the gel spots in a vacuum concentrator (e.g., Speedvac) for 5–10 min.

3.2.1.4. Digest Proteins in Gel and Extract Peptides from Gel Spots

1. The dried gel spots are swelled in 10 μL of 20 ng/ μL trypsin on ice and incubated for 30–45 min.
2. Next, 10 μL of 100 mM NH_4HCO_3 is added and the digestion is allowed at 37°C overnight.
3. Next day, 20 μL of H_2O is added to each tube and the supernatant is transferred to a new 1.5 mL micro-centrifuge tube.
4. 30 μL of 50% $\text{CH}_3\text{CN}/0.1\%$ TFA is added to the gel spots. After shaking at 150 rpm for 30 min, the supernatant is removed and added to the previous supernatant collected in step 3. This step is repeated twice.
5. Finally, the extracted peptides are dried in a Speedvac and redissolved in 10 μL of LC mobile phase A for LC-MS/MS analysis.

3.2.2. LC-MS/MS

The peptide samples are analyzed using nano-LC (Eksigent Technology) with linear ion trap MS (LTQ XL, Thermo-Fisher Scientific). The LC separation is performed with a PepMap C18 column (75 $\mu\text{m} \times 150\text{ mm}$; particle size 3 μm , Dionex, Sunnyvale, CA) at a flow rate of 400 nL/min. The LC gradient elution started from 15% mobile phase B to 95% mobile phase B within 30 min and then held at 95% B for 20 min and finally put back immediately to 5% mobile phase B for a 15 min column equilibration. The eluent is introduced directly to the LTQ mass spectrometer via electrospray using a PicoTip emitter (tip inner diameter, 10 μm). Each full MS scan is followed by 5 data-dependent MS/MS scans on the most intense ions at a 35% normalized collision energy.

3.2.3. SEQUEST Database Searching

The acquired MS/MS data are searched against the human IPI (International Protein Index) database version 3.32 using the SEQUEST algorithm (Thermo-Fisher Scientific).

1. The search parameters are as follows: enzyme limit: partially enzymatic cleaves at either end; missed cleavage sites: 2; precursor peptide tolerance: 2.000 AMU; fragment ion tolerance: 1.000 AMU; modifications: carbamidomethylated cysteine (+57) and oxidized methionines (+16).
2. The filter parameters are as follows: delta CN = 0.100; RSp = 1; Xcorr vs. charge state = 2.0, 2.5, 3.0; peptide probability = 0.001; number of different peptides = 2.

3.3. Western Blot Analysis of Saliva Proteins

3.3.1. SDS-PAGE

First the total protein concentration of each individual sample is determined (*see Section 3.1.1*). Samples with same amount of proteins (for example, 20 μg) as well as See Blue Plus2 pre-stained protein standards are then loaded into gel wells. SDS-PAGE is run on 12% NuPAGE minigels (1 mm $\times$ 12 wells) in MES SDS running buffer at 100 V for 1–1.5 h (*see Note 3*).

3.3.2. Western Blotting

PAGE gels are removed from gel cassettes and rinsed gently in water. Then the gel is placed on the nitrocellulose membrane of an iBlot anode stack, followed by a pre-wetted filter paper and the cathode stack. The blot transfer is completed at 23 V within 6 min and the membrane is kept in a container for the following blotting steps.

1. *Blocking*: 5% NFDm/TBST(1 $\times$), 2 h at room temperature with gentle shaking.
2. *Washing*: TBST(1 $\times$), 30 min $\times$ 3 times with gentle shaking.
3. *Primary antibody incubation*: Primary antibody at the recommended concentration is prepared with 5%

NFDM/TBST(1×). Incubate for 2 h at room temperature with gentle shaking.

4. *Washing*: TBST(1×), 30 min × 3 times with gentle shaking.
5. *Secondary antibody incubation*: Secondary antibody is prepared with 5% NFDM/TBST(1×). Incubation for 1 h at room temperature with gentle shaking.
6. *Washing*: Washed with 1× TBST, 30 min × 3 times with gentle shaking.
7. *Detection*: The A and B solutions of the ECL detection kit are mixed at a ratio of 1:1. The mixture solution is applied to the membrane blots carefully and the film is immediately developed in a dark room. The film is then scanned and analyzed using the Scion Image software.

3.4. Shotgun Proteomics

3.4.1. LC Pre-fractionation of Whole Saliva Proteins

Whole saliva samples (100 µg proteins in total each) are separated by HP1100 LC system (Agilent Technologies) using a Vydac C4 reversed-phase column (particle size, 5 µm; 250 × 2.1 mm inner diameter; The Nest Group, Inc.) at a flow rate of 250 µL/min (*see Note 4*). The LC gradient elution started from 5% mobile phase B to 85% mobile phase B within 40 min and then held at 85% B for 25 min. In total, 35 LC fractions were collected for each whole saliva sample (1 fraction per min).

3.4.2. In-Solution Digestion

1. The proteins in each LC fraction are reduced with DTT (10 mM, 1 h), alkylated with iodoacetamide (55 mM, 1 h), and digested by trypsin (60 ng trypsin for each fraction) at 37°C for overnight.
2. The resulting peptide digests are dried, reconstituted in 0.1% formic acid, and then analyzed by capillary LC-quadrupole time-of-flight (QqTOF) MS.

3.4.3. Capillary LC-QqTOF MS for Peptide Analysis

LC-MS/MS analysis is performed using a LC Packings nano-LC system (Dionex) with a nanoelectrospray interface (Protana) and QqTOF mass spectrometer (QSTAR XL, Applied Biosystems). The samples are first loaded onto a home-packed C18 precolumn (300 µm × 1 mm; particle size, 5 µm) and then injected onto a LC Packings PepMap C18 column (75 µm × 150 mm; particle size, 5 µm) for nano-LC separation at a flow rate of 250 nL/min. The LC gradient elution started from 5% mobile phase B to 60% mobile phase B within 55 min and then held at 95% B for 15 min and finally back immediately to 5% mobile phase A for a 15 min column equilibration. A New Objective PicoTip (tip inner diameter, 8 µm) is used for electrospraying with the voltage at 1,850 V for online MS and MS/MS analyses (*see Note 5*).

3.4.4. MASCOT Database Searching

MASCOT (version 1.9; Matrix Science) is used for database searches with search parameters containing the following modifications: carbamidomethylated cysteine (+57) and oxidized methionines (+16). All searches are performed against the human IPI database with one missed cleavage allowed and a mass tolerance of 0.3 Da for both precursor and product ions. A Mascot score with $P < 0.05$ is considered a significant match of a peptide (*see* **Note 6**).

4. Notes

1. Sample preparation is critical for proteomic analysis. A consistent protocol for sample collection and processing should be used for all saliva samples. A saliva sample must be centrifuged (typically 2,600g for 15 min) to remove cell pellets and debris in order to collect the supernatant for proteomic analysis.
2. To discover and validate protein biomarkers in saliva, a patient/control sample set must be well matched in terms of age, gender, ethnicity, and important risk factors such as smoking history for oral or lung cancer.
3. The viscosity of saliva is high and may be different between samples. Prior to Western blot analysis, saliva samples must be denatured well in order to minimize the background. For validation of protein biomarkers using Western blotting, each individual patient sample should be tested side by side with its healthy control on the same gel.
4. C4 reversed-phase column is often used for LC separation of proteins whereas C18 column is often used for separation of peptides. Besides reversed-phase LC, other separation techniques including ZOOM IEF, size-exclusion LC, free-flow electrophoresis, ultracentrifugation, and ion-exchange LC can be used to pre-fractionate whole saliva proteins.
5. Large-scale identification of whole saliva proteins can be accomplished using MudPIT (Multidimensional Protein Identification Technology), which is a powerful technique for the separation and identification of complex protein and peptide mixtures (21). MudPIT is based on direction in-solution digestion of proteins followed by 2D liquid chromatography (strong cation exchange and reversed-phase LC) and MS/MS for identification of the resulting peptides.
6. Regarding comprehensive identification of saliva proteins using shotgun proteomics, false-positive rates can be

calculated by multiplying the number of false-positive identifications (hits to the decoy database constructed from randomized sequences) and dividing by the number of total identifications.

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References

1. Tabak, L. A. (2001) A revolution in biomedical assessment: the development of salivary diagnostics. *J. Dent. Educ.* **65**, 1335–1339.
2. Mandel, I. D. (1990) The diagnostic uses of saliva. *J. Oral Pathol. Med.* **19**, 119–125.
3. Yao, Y., Berg, E. A., Costello, C. E., Troxler, R. F., and Oppenheim, F. G. (2003) Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. *J. Biol. Chem.* **278**, 5300–5308.
4. Lupi, A., Messana, I., Denotti, G., Schinina, M. E., Gambarini, G., Fadda, M. B., Vitali, A., Cabras, T., Piras, V., Patamia, M., Cordaro, M., Giardina, B., and Castagnola, M. (2003) Identification of the human salivary cystatin complex by the coupling of high-performance liquid chromatography and ion-trap mass spectrometry. *Proteomics* **3**, 461–467.
5. Wilmarth, P. A., Riviere, M. A., Rustvold, D. L., Lauten, J. D., Madden, T. E., and David, L. L. (2004) Two-dimensional liquid chromatography study of the human whole saliva proteome. *J. Proteome Res.* **3**, 1017–1023.
6. Hu, S., Xie, Y., Ramachandran, P., Loo, R. R., Li, Y., Loo, J. A., and Wong, D. T. (2005) Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. *Proteomics* **5**, 1714–1728.
7. Xie, H., Rhodus, N. L., Griffin, R. J., Carlis, J. V., and Griffin, T. J. (2005) A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. *Mol. Cell Proteomics* **4**, 1826–1830.
8. Guo, T., Rudnick, P. A., Wang, W., Lee, C. S., Devoe, D. L., and Balgley, B. M. (2006) Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. *J. Proteome Res.* **5**, 1469–1478.
9. Denny, P., Hagen, F. K., Hardt, M., Liao, L., Yan, W., Arellanno, M., Bassilian, S., Bedi, G. S., Boonthueung, P., Cociorva, D., Delahunty, C. M., Denny, T., Dunsmore, J., Faull, K. F., Gilligan, J., Gonzalez-Begne, M., Halgand, F., Hall, S. C., Han, X., Henson, B., Hewel, J., Hu, S., Jeffrey, S., Jiang, J., Loo, J. A., Loo, R. R., Malamud, D., Melvin, J. E., Miroshnychenko, O., Navazesh, M., Niles, R., Park, S. K., Prakobphol, A., Ramachandran, P., Richert, M., Robinson, S., Sondej, M., Souda, P., Sullivan, M. A., Takashima, J., Than, S., Wang, J., Whitelegge, J. P., Witkowska, H. E., Wolinsky, L., Xie, Y., Xu, T., Yu, W., Ytterberg, J., Wong, D. T., Yates, J. R., 3rd, and Fisher, S. J. (2008) The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *J. Proteome Res.* **7**, 1994–2006.
10. Messana, I., Cabras, T., Pisano, E., Sanna, M. T., Olianias, A., Manconi, B., Pellegrini, M., Paludetti, G., Scarano, E., Fiorita, A., Agostino, S., Contucci, A. M., Calò, L., Picciotti, P. M., Manni, A., Bennick, A., Vitali, A., Fanali, C., Inzitari, R., and Castagnola, M. (2008) Trafficking and postsecretory events responsible for the formation of secreted human salivary peptides: a proteomics approach. *Mol. Cell Proteomics* **7**, 911–926.

11. Castagnola, M., Inzitari, R., Rossetti, D. V., Olmi, C., Cabras, T., Piras, V., Nicolussi, P., Sanna, M. T., Pellegrini, M., Giardina, B., and Messina, I. (2004) A cascade of 24 histatins (histatin 3 fragments) in human saliva. Suggestions for a pre-secretory sequential cleavage pathway. *J. Biol. Chem.* **279**, 41436–41443.
12. Cabras, T., Fanali, C., Monteiro, J. A., Amado, F., Inzitari, R., Desiderio, C., Scarano, E., Giardina, B., Castagnola, M., and Messina, I. (2007) Tyrosine polysulfation of human salivary histatin 1. A post-translational modification specific of the submandibular gland. *J. Proteome Res.* **6**, 2472–2480.
13. Ramachandran, P., Boonthung, P., Xie, Y., Sondej, M., Wong, D. T., and Loo, J. A. (2006) Identification of *N*-linked glycoproteins in human saliva by glycoprotein capture and mass spectrometry. *J. Proteome Res.* **5**, 1493–1503.
14. Yan, W., Apweiler, R., Balgley, B. M., Boonthung, P., Bundy, J. L., Cargile, B. J., Cole, S., Fang, X., Gonzalez-Begne, M., Griffin, T. L., Hagen, F., Hu, S., Wolinsky, L. E., Lee, C. S., Malamud, D., Melvin, J. E., Menon, R., Mueller, M., Qiao, R., Rhodus, N. L., Sevinsky, J. R., States, D., Stephenson, J. L., Jr., Than, S., Yates, J. R., III, Yu, W., Xie, H., Xie, Y., Omenn, G. S., Loo, J. A., and Wong, D. T. (2009) Systematic comparison of the human saliva and plasma proteomes. *Proteomics/Clin. Appl.* **3**, 116–134.
15. Ryu, O. H., Atkinson, J. C., Hoehn, G. T., Illei, G. G., and Hart, T. C. (2006) Identification of parotid salivary biomarkers in Sjogren's syndrome by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and two-dimensional difference gel electrophoresis. *Rheumatology (Oxford)*. **45**, 1077–1086.
16. Hu, S., Wang, J., Meijer, J., Ieong, S., Xie, Y., Yu, T., Zhou, H., Henry, S., Vissink, A., Pijpe, J., Kallenberg, C., Elashoff, D., Loo, J. A., and Wong, D. T. (2007) Salivary proteomic and genomic biomarkers for primary Sjögren's syndrome. *Arthritis Rheum.* **56**, 3588–3600.
17. Peluso, G., De Santis, M., Inzitari, R., Fanali, C., Cabras, T., Messina, I., Castagnola, M., and Ferraccioli, G. F. (2007) Proteomic study of salivary peptides and proteins in patients with Sjögren's syndrome before and after pilocarpine treatment. *Arthritis Rheum.* **56**, 2216–2222.
18. Hu, S., Arellano, M., Boonthung, P., Wang, J., Zhou, H., Jiang, J., Elashoff, D., Wei, R., Loo, J. A., and Wong, D. T. (2008) Salivary proteomics for oral cancer biomarker discovery. *Clin. Cancer Res.* **14**, 6246–6252.
19. Streckfus, C. F., Mayorga-Wark, O., Arreola, D., Edwards, C., Bigler, L., and Dubinsky, W. P. (2008) Breast cancer related proteins are present in saliva and are modulated secondary to ductal carcinoma in situ of the breast. *Cancer Invest.* **26**, 159–167.
20. Xie, H., Onsongo, G., Popko, J., de Jong, E. P., Cao, J., Carlis, J. V., Griffin, R. J., Rhodus, N. L., and Griffin, T. J. (2008) Proteomics analysis of cells in whole saliva from oral cancer patients via value-added three-dimensional peptide fractionation and tandem mass spectrometry. *Mol. Cell Proteomics.* **7**, 486–498.
21. Wolters, D. A., Washburn, M. P., and Yates, J. R., 3rd. (2001) An automated multidimensional protein identification technology for shotgun proteomics. *Anal. Chem.* **73**, 5683–5690.

Chapter 4

Transcriptomic Analyses of Saliva

Viswanathan Palanisamy and David T. Wong

Abstract

Salivary biomarkers for diagnostic and prognostic assessments have become increasingly well established in recent years. Salivary mRNA transcriptomic analyses create a new paradigm in the emerging field for noninvasive molecular diagnosis. In this chapter, we will overview the development of sensitive and robust microarray and multiplex quantitative reverse transcriptase-PCR assays for the discovery and validation of mRNA biomarkers in human saliva. Total RNA isolated from human saliva is used for microarray profiling through Human Genome U133 Plus 2.0 and Exon 1.0 ST array platforms. A universal RNA linear amplification strategy was used to amplify RNA from nanogram scale followed by reverse transcription-PCR reaction, cleaned up enzymatically, and validated by quantitative PCR. Further, the integrity of RNA can be analyzed by the Agilent Bioanalyzer and quantified using a Nanodrop microvolume spectrophotometer. Using these invaluable technical tools, one can identify thousands of mRNA species in saliva. These methods indicate that salivary mRNA provides an efficient medium for biomarker discovery in oral and systemic diseases detection.

Key words: Saliva, mRNA profiling, biomarkers, oral cancer, microarray profile, mRNA stability.

1. Introduction

Messenger RNA in human saliva is an emerging field for non-invasive diagnostic applications. The discoveries of saliva-derived mRNA in normal and oral cancer patients (1–3) and other forensic applications (4, 5) have opened up a new field for studying gene expression noninvasively. Our laboratory has extensively studied microarray-based gene profiling followed by real-time quantitative reverse transcription-PCR (RT-PCR) for saliva RNA detection. The existence of saliva RNA is a remarkable finding because RNA is more labile than DNA and particularly because ribonucleases are known to be present in saliva (6) to degrade

RNA molecules. We have identified certain macromolecules associated with saliva RNA which may protect it against ribonucleases (6). The other possible scenarios for protection of salivary RNA may be when it is complexed to lipids, proteins, lipoproteins, or phospholipids as in serum (7, 8) or when protected within apoptotic bodies (9) or other vesicular structures. Therefore, RNA in the saliva may not be as fragile as it was previously assumed to be.

Although a variety of platforms are available for mRNA expression analysis, currently the Affymetrix U133 Plus 2.0 (U133 Plus 2.0) and Human Exon 1.0 ST (HuEx) platforms are noteworthy. Microarray profiling followed by quantitative PCR analysis is widely accepted for biomarker discoveries. RNA is detectable in saliva but little is known about its integrity and stability. Theoretically, to maintain RNA integrity, the time and steps between collection and RNA extraction should be reduced to the minimum. Methods such as snap-freezing of saliva or the addition of a stabilizing reagent have also been used to reduce the chance of RNA degradation. Of importance are detailed studies reported by our group (10, 11), on the extraction and stability of saliva RNA, and these will be the primary focus of this methods entity.

The purpose of this chapter is to provide simple and reliable methods for isolating and profiling saliva RNA. Here, we describe a detailed methodology for extraction, amplification, and gene expression profiling of saliva RNA using microarray and PCR techniques. Saliva contains a multitude of proteins and DNA; therefore, before performing any interpretation about mRNA species, it is critical to ensure that the isolated RNA is human RNA and not other contaminating nucleic acids. The first part describes the most common protocols used to isolate RNA from human saliva and the second part describes different microarray platforms for profiling and assessing the quality of the isolated saliva RNA. The isolation of RNA from saliva involves few centrifugation and incubation steps. Of note is that commercial RNA extraction kits are available to extract RNA from saliva (for example, RNeasy Protect Saliva Mini Kit; QIAGEN, USA and Oragene RNA kit; DNA Genotek, Ottawa, Ontario, Canada). Here we describe a robust, highly reproducible method of RNA extraction using commercial reagents, designed for isolating RNA in laboratory settings. An extra washing and incubation step in extraction of RNA from a silica column provides extremely pure mRNA for downstream applications. The final part of the unit describes methods used for gene profiling of saliva RNA using Affymetrix U133 Plus 2.0 and HuEx array microarray platforms.

We have combined universal full-length linear amplification of mRNA, a microarray platform with U133 plus 2.0 and all exon-level resolution, and comprehensive q-PCR validation. The comprehensiveness and advantages of our approach are

demonstrated by the detection of many new transcript fragments and most notably the fragmented RNA can also be detected. The demonstration of the ability to identify fragmented RNA from saliva exon profiles provides early evidence that salivary exon profiles may have clinical value in disease diagnosis. This approach has provided a comprehensive method that is also applicable for biomarker studies of other body fluids and clinical samples containing fragmented RNAs.

2. Materials

2.1. Saliva Collection and Processing

1. 50 mL of sterile tube and styrofoam cup
2. Crushed ice
3. Distilled water
4. Laboratory vortex
5. Refrigerated bench top centrifuge with 50 mL tube adapters
6. RNase inhibitor (Ambion Inc., Austin, TX, USA)

2.2. RNA Isolation

1. RNeasy Micro Kit, QIAGEN, Valencia, CA, USA
2. Absolute ethanol
3. RNase-free water
4. Instrument: Nanodrop 3300 microvolume spectrophotometer (Thermo Scientific, USA).

2.3. Target cRNA Preparation

1. RiboAmp[®] Plus kit (Molecular devices, Sunnyvale, CA USA)
2. Affymetrix RNA labeling kit (Affymetrix, Santa Clara, USA)
3. GeneChip[®] Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA)
4. 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA)
5. RNase-free water
6. Absolute ethanol
7. PCR machine

2.4. U133 Plus 2.0 Microarray Analysis

1. Affymetrix Human Genome U133 plus 2.0 Arrayer
2. Microarray Suite (MAS) software (Affymetrix).
3. Computer

2.5. All Exon Array and Data Processing

1. WT cDNA Synthesis Kit (Affymetrix)
2. WT Terminal Labeling Kit (Affymetrix).

3. Gene Chip Human Exon 1.0 ST arrays (Affymetrix)
4. Computer
5. Exon Array Computational Tool (ExACT) (Affymetrix)

2.6. Quantitative Gene Expression Analysis by q-PCR

1. ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA)
2. MuLV Reverse Transcriptase (Applied Biosystems)
3. Power SYBR Green PCRMix (Applied Biosystems)
4. Gene-specific primers (e.g., synthesized by Sigma-Genosys (Woodlands, TX, USA))
5. ABI 7500 Fast system software (Applied Biosystems)

2.7. Statistical Analysis

1. Software R Codes: <http://www.r-project.org>

3. Methods

Saliva collection from human subjects has to be approved by the Institutional Review Board. The inclusion criteria we used for normal subject selection were age ≥ 30 years and no history of malignancy, immunodeficiency, autoimmune disorders, hepatitis, HIV infection, or smoking. Our oral cancer patients were recruited with documented primary T1 or T2 oral squamous cell carcinoma. All of the patients had received diagnoses of primary disease or not received any prior treatment in the form of chemotherapy, radiotherapy, surgery, or alternative remedies.

3.1. Saliva Collection and Processing

1. Unstimulated saliva samples should be collected between 9 a.m. and 10 a.m. in accordance with published protocols (12). Ask subjects to refrain from eating, drinking, smoking, or oral hygiene procedures for at least an hour before collection.
2. The subjects should be instructed to rinse their mouth thoroughly with deionized water prior to the collection and to void the mouth of saliva. The subject should be seated comfortably with eyes open, head tilted slightly forward, and (for unstimulated saliva collection) instructed to rest for 5 min and to minimize orofacial movements. The subject should allow the saliva to accumulate in the floor of the mouth before spitting it into a preweighed or graduated test tube every 60 s. 5 min of collection time is usually enough for a sufficient amount of saliva (~ 5 mL) for analysis.
3. Following collection, centrifuge saliva samples at $2,600g$ for 15 min at 4°C . Saliva supernatant will then be separated

from the cellular phase. RNase inhibitor (500 units/mL) is added to the cell-free saliva supernatant (*see* **Notes 1, 2, and 3**).

3.2. RNA Isolation from Cell-Free Saliva

1. Isolate RNA from the cell-free saliva supernatant according to the modified protocol from the manufacturer (RNeasy Micro Kit) (*see* **Note 4**).
2. Saliva (300 μ L), mixed well with 700 μ L of RLT buffer (supplied with the kit; contains guanidine thiocyanate), is incubated at room temperature for 10 min with occasional vortexing.
3. Next, absolute ethanol (500 μ L) is added, and the solution passed through a silica column (RNeasy MinElute spin column) by centrifugation at $\geq 8,000g$ for 1 min.
4. Wash the column with 350 μ L of buffer RW1 (supplied with the kit; contains ethanol) followed by incubation at room temperature for 15 min with 10 μ L RNase-free DNase (supplied with RNeasy Micro Kit) and 70 μ L of buffer RDD (supplied with the kit).
5. Wash the column with 350 μ L of buffer RW1 followed by 500 μ L of buffer RPE (supplied with RNeasy Micro Kit).
6. Wash the column once with 500 μ L of 80% ethanol and centrifuge the contents. Discard the flow through.
7. Elute the RNA with 30 μ L RNase-free water at 13,000 g for 2 min.
8. Measure the quantity of RNA using the Nanodrop 3300 microvolume spectrophotometer.

3.3. Target cRNA Preparation

1. Isolated RNA is subjected to linear amplification using RiboAmp[®] Plus according to the protocol from the manufacturer.
2. Round one of RNA amplification utilizes 10–11 μ L of eluted RNA for first-strand cDNA synthesis. Following round one, second-strand synthesis is carried out using the supplied primers from the kit, followed by in vitro transcription (IVT).
3. To maximize the RNA yield, a second round of amplification has to be carried out using the supplied primers in reverse order. After this round of second-strand synthesis (1.5 rounds of RNA amplification), the Affymetrix RNA Labeling kit can be used for the second round of IVT to biotinylate the cRNA product.
4. The labeled cRNA is purified with the use of the GeneChip[®] Sample Cleanup Module.

5. Small aliquots from each of the isolation and amplification steps are used to assess the quality by RT-PCR. The quality of the fragmented cRNA prepared as described in (13) is assessed by capillary electrophoresis with the 2100 Bioanalyzer.

3.4. U133 Plus 2.0 Microarray Analysis

1. The Affymetrix Human Genome U133 plus 2.0 Array, which contains 54,000 probe sets representing ~38,500 genes (i.e., each gene may be represented by more than one probe set), is used for gene expression profiling.
2. The array data can be normalized and analyzed by means of Microarray Suite (MAS) software (Affymetrix).
3. A detection *P*-value will be obtained for each probe set. Any probe set with a *P* value <0.001 and an intensity value >200 is assigned as “present,” indicating that the matching gene transcript is reliably detected (*see Note 5*).
4. The total number of present probe sets on each array can be obtained and the percentage (%) of present genes calculated.
5. Functional classification is performed on selected genes (present on all 10 arrays, *P* < 0.01) by means of the Gene Ontology Mining Tool (<http://www.netaffx.com>).

3.5. All Exon Array and Data Processing

1. Single-stranded cDNA is generated from the amplified cRNA with the WT cDNA Synthesis Kit then fragmented and labeled with the WT Terminal Labeling Kit.
2. Samples are hybridized with GeneChip Human Exon 1.0 ST Arrays and scanned at the Microarray Facility. Raw data can be processed with the Exon Array Computational Tool (ExACT) (Affymetrix) for background correction and normalization.
3. For example, the salivary mRNA Exon array raw data has been deposited in the Gene Expression Omnibus (GEO) database under series accession no. GSE7760.

3.6. Quantitative Gene Expression Analysis by q-PCR

1. q-PCR can be performed with use of an ABI 7500 real-time PCR System.
2. 2 μ L aliquot of the isolated salivary RNA (without amplification) is reverse transcribed into cDNA by means of MuLV Reverse Transcriptase.
3. The resulting cDNA (3 μ L) is used for PCR amplification with Power SYBR Green PCRmix.
4. The primers were synthesized by Sigma-Genosys and Table 4.1 shows the primers used and validated for these studies.

Table 4.1
Selection of primers used for quantitative PCR validation of selected nine transcripts in saliva ($n = 64$)

Gene symbol	Primer sequence (5'–3')	Validated ^a	<i>P</i> value	Mean fold increase
<i>DUSP1</i>	F: CCTACCAGTATTATTCCTGACG R: TTGTGAAGGCAGACACCTACAC	Yes	0.039	2.60
<i>H3F3A</i>	F: AAAGCACCCAGGAAGCAAC R: GCGAATCAGAAGTTCAGTGGAC	Yes	0.011	5.61
<i>IL1B</i>	F: GTGCTGAATGTGGACTCAATCC R: ACCCTAAGGCAGGCAGTTG	Yes	0.005	5.48
<i>IL8</i>	F: GAGGGTTGTGGAGAAGTTTTTG R: CTGGCATCTTCACTGATTCTTG	Yes	0.000	24.3
<i>OAZ1</i>	F: AGAGAGAGTCTTCGGGAGAGG R: AGATGAGCGAGTCTACGGTTC	Yes	0.009	2.82
<i>S100P</i>	F: GAGTTCATCGTGTTCGTGGCTG R: CTCCAGGGCATCATTTGAGTCC	Yes	0.003	4.88
<i>SAT</i>	F: CCAGTGAAGAGGGTTGGAGAC R: TGGAGGTTGTCATCTACAGCAG	Yes	0.005	2.98
<i>GADD45B</i>	F: TGATGAATGTGGACCCAGAC R: GAGCGTGAAGTGGATTTC	No	0.116	
<i>RGS2</i>	F: CCTGCCATAAAGACTGACCTTG R: GCTTCCTGATTCACTACCCAAC	No	0.149	

NOTE. q-PCR was performed to validate the microarray findings on an enlarged sample size including saliva from 32 patients with OSCC and 32 matched control subjects. Nine potential salivary mRNA biomarkers were selected from the 17 candidates shown in Table 4.1. Seven of them were validated by q-PCR ($P < 0.05$). Sample includes 32 saliva from OSCC patients and 32 from matched normal subjects.

^aWilcoxon's signed rank test: if $P < 0.05$, validated (Yes); if $P > 0.05$, not validated (No).

5. All reactions have to be performed in triplicate, with conditions customized for the specific PCR products. The initial amount of cDNA of a particular template is extrapolated from a standard curve with the use of 7500 Fast system software.
6. The detailed procedure for quantification by standard curve has been previously described (14).

3.7. Statistical Analysis

1. Data analysis and statistical evaluations can be performed with customized R codes (version 2.3.1, <http://www.r-project.org/>).
2. Define a probe set as present when it has a P value < 0.001 and an intensity value > 200 . These criteria were assessed by the results of preliminary experiments.
3. The SECT includes all probe sets present in microarrays. In addition, you can refine the SECT to remove GC-rich (i.e.,

$\geq 80\%$) probe sets. The concordance between the SECT and the normal salivary core transcriptome (NSCT) was evaluated assuming hypergeometric distributions.

4. For q-PCR data, we normalized all transcript abundances to the three genes in the saliva internal reference (SIR): *ANXA2* (annexin A2), *RPL37* (ribosomal protein L37), and *SI00A* (see **Note 6**).
5. Specifically, you can subtract the mean of the SIR genes' cycle threshold (C_T) values from all of the raw C_T values and use the resulting ΔC_T values to represent the relative abundance of the transcripts (15).

4. Notes

1. Supernatant phase of saliva has to be used to extract enough total RNA for cDNA preparations.
2. The quality of RNA could meet the demand for PCR, q-PCR, and microarray assays. It is always better to add RNase inhibitors to freshly collected oral fluids followed by ultra-low-temperature storage (-80°C).
3. Importantly, the conditions for separating the pellet and saliva supernatant have to be optimized to avoid mechanical rupture of cellular elements which would contribute to the RNA detected in the fluidic cell-free phase (16).
4. There are several commercial extraction kits available, but notably QIAGEN's RNA protect saliva and DNA Genotek's Oragene RNA are good examples for rapid isolation of RNA from saliva.
5. The guide to interpreting detection and intensity P -values is detailed in the Affymetrix Technical Note: New statistical algorithms for monitoring gene expression on GeneChip® Probe Arrays (http://www.affymetrix.com/support/technical/technotes/statistical_algorithms_technote.pdf).
6. The three genes representing the Salivary Internal Reference are amplified using the following primers:
 - (a) *ANXA2* – Forward primer: TCCCTGTACTATTAT-ATCCAGCAA, reverse primer: TTCTGGTAGTCGCC-CTTAGTGT
 - (b) *RPL37* – Forward primer: GAAATACCACCGGAA-CTGGTC, reverse primer: GAATCCATGCCTGAA-TCTGC
 - (c) *SI00A8* – Forward primer: TGCTAGAGACCGAGTG-TCCTCAG, reverse primer: CATCAGTGTGATAT-CCAACTCTTTGA

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References

- Li, Y., St John, M. A., Zhou, X., Kim, Y., Sinha, U., Jordan, R. C., Eisele, D., Abemayor, E., Elashoff, D., Park, N. H., and Wong, D. T. (2004) Salivary transcriptome diagnostics for oral cancer detection. *Clin. Cancer Res.* **10**, 8442–8450.
- Li, Y., Zhou, X., St John, M. A., and Wong, D. T. (2004) RNA profiling of cell-free saliva using microarray technology. *J. Dent. Res.* **83**, 199–203.
- Hu, Z., Zimmermann, B. G., Zhou, H., Wang, J., Henson, B. S., Yu, W., Elashoff, D., Krupp, G., and Wong, D. T. (2008) Exon-level expression profiling: a comprehensive transcriptome analysis of oral fluids. *Clin. Chem.* **54**, 824–832.
- Juusola, J., and Ballantyne, J. (2005) Multiplex mRNA profiling for the identification of body fluids. *Forensic Sci. Int.* **152**, 1–12.
- Juusola, J., and Ballantyne, J. (2007) mRNA profiling for body fluid identification by multiplex quantitative RT-PCR. *J. Forensic Sci.* **52**, 1252–1262.
- Park, N. J., Li, Y., Yu, T., Brinkman, B. M., and Wong, D. T. (2006) Characterization of RNA in saliva. *Clin. Chem.* **52**, 988–994.
- Rosi, A., Guidoni, L., Luciani, A. M., Mariutti, G., and Viti, V. (1988) RNA-lipid complexes released from the plasma membrane of human colon carcinoma cells. *Cancer Lett.* **39**, 153–160.
- Whitelegge, J. P., Zabrouskov, V., Halgand, F., Souda, P., Bassilian, S., Yan, W., Wolinsky, L., Loo, J. A., Wong, D. T., and Faull, K. F. (2007) Protein-sequence polymorphisms and post-translational modifications in proteins from human saliva using top-down Fourier-transform ion cyclotron resonance mass spectrometry. *Int. J. Mass. Spectrom.* **268**, 190–197.
- Halicka, H. D., Bedner, E., and Darzynkiewicz, Z. (2000) Segregation of RNA and separate packaging of DNA and RNA in apoptotic bodies during apoptosis. *Exp. Cell Res.* **260**, 248–256.
- Jiang, J., Park, N. J., Hu, S., and Wong, D. T. (2008) A universal pre-analytic solution for concurrent stabilization of salivary proteins, RNA and DNA at ambient temperature. *Arch. Oral Biol.* **54**, 268–273.
- Park, N. J., Yu, T., Nabili, V., Brinkman, B. M., Henry, S., Wang, J., and Wong, D. T. (2006) RNAprotect saliva: an optimal room-temperature stabilization reagent for the salivary transcriptome. *Clin. Chem.* **52**, 2303–2304.
- Navazesh, M. (1993) Methods for collecting saliva. *Ann. NY Acad. Sci.* **694**, 72–77.
- Kelly, J. J., Chernov, B. K., Tovstakov, I., Mirzabekov, A. D., and Bavykin, S. G. (2002) Radical-generating coordination complexes as tools for rapid and effective fragmentation and fluorescent labeling of nucleic acids for microchip hybridization. *Anal. Biochem.* **311**, 103–118.
- Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods.* **25**, 402–408.
- Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **3**, RESEARCH0034.
- St John, M. A., Li, Y., Zhou, X., Denny, P., Ho, C. M., Montemagno, C., Shi, W., Qi, F., Wu, B., Sinha, U., Jordan, R., Wolinsky, L., Park, N. H., Liu, H., Abemayor, E., and Wong, D. T. (2004) Interleukin 6 and interleukin 8 as potential biomarkers for oral cavity and oropharyngeal squamous cell carcinoma. *Arch. Otolaryngol. Head Neck Surg.* **130**, 929–935.

Section II

Oral Microbiology

Chapter 5

The Oral Microbiota: General Overview, Taxonomy, and Nucleic Acid Techniques

José F. Siqueira Jr. and Isabela N. Rôças

Abstract

Application of nucleic acid technology to the analysis of the bacterial diversity in the oral cavity in conditions of health and disease has not only confirmed the findings from early culture studies but also significantly expanded the list of oral inhabitants and candidate pathogens associated with the major oral diseases. Over 800 bacterial distinct species-level taxa have been detected in the oral cavity and recent studies using high-throughput technology suggest that the breadth of bacterial diversity can be much larger. This chapter provides an overview of the diversity and taxonomy of oral bacteria. Emphasis is also given on nucleic acid technologies that have been widely used for the study of the oral microbiota.

Key words: Oral microbiology, molecular biology methods, taxonomy.

1. Introduction

The microbiota colonizing the oral cavity is composed of diverse groups of bacterial species, each one possessing its specific nutritional and physico-chemical requirements. For oral bacteria to be successfully grown in the laboratory, culturing conditions have to be adjusted to suit their varied requirements (1). A high diversity of bacterial species has been disclosed in the oral cavity by culture, but early microscopy studies had already suggested that roughly one-half of the oral microbiota cannot be cultivated in vitro (2). Introduction of culture-independent nucleic acid methods to the analysis of oral bacterial diversity has not only confirmed this picture revealed by microscopic studies, but also demonstrated a still broader and more diverse spectrum of extant oral bacteria.

This chapter provides an overview of bacterial diversity and taxonomy in the oral cavity under healthy and diseased conditions. Nucleic acid technologies that have been widely used for the study of oral bacteria are also highlighted.

2. Diversity and Taxonomy of Oral Bacteria

Data from culture and molecular studies have collectively revealed that almost 800 distinct bacterial species-level taxa may be able to live in the human oral cavity (3). Not all of them are present in the same individual at any one time and a particular individual can harbor about 100–200 taxa in his/her mouth. Whereas some species are common to different oral sites, the majority of species are selective for a particular site (4). Of the >800 species, over 50% remain to be cultivated and fully characterized. This raises the interesting possibility that as-yet-uncultivated and uncharacterized species that have passed unnoticed by culturing studies may actually play an important ecological, beneficial, or pathogenic role in the oral cavity.

At a broad taxonomic level, bacteria detected in the oral cavity belong to 13 separate phyla. The majority of oral species-level taxa fall into the phyla Firmicutes, Fusobacteria, Bacteroidetes, Actinobacteria, Proteobacteria, Spirochaetes, Synergistes, and TM7, while representatives of the phyla SR1, Chloroflexi, Cyanobacteria, Deinococcus, and Acidobacteria have been sporadically reported (5–9). This number may be even higher as a recent study using DNA microarray technology suggested that members of four other phyla (Aquificae, Nitrospira, Planctomycetes, and Thermomicrobia) can have oral representatives, even though none has been identified (10). Indeed, we may have so far just scratched the surface. A recent study using pyrosequencing, a relatively new high-throughput molecular approach that allows for extensive sequencing of microbial populations, explored the composition of the microbiota in saliva and dental plaque by targeting the V6 region of the 16S rRNA gene. Findings revealed about 5,600 and 10,000 species-level phylotypes representing 22 phyla in saliva and plaque, respectively (11). The estimated number of oral phylotypes is about 20,000, which is considerably higher when compared to previous culture and clone library studies.

3. Refined Bacterial Taxonomy Associated with Oral Diseases

Caries. Cultivable species of *Streptococcus*, *Lactobacillus*, and *Actinomyces* are closely associated with the etiopathogenesis of different forms and stages of caries (12, 13). However, recent

nucleic acid approaches have demonstrated that the diversity of the microbiota associated with caries is far greater than anticipated. Overall, about 40–60% of the microbiota occurring in caries lesions is made up of as-yet-uncultivated species (14–18). As-yet-uncultivated phylotypes or uncharacterized strains of *Bifidobacterium*, *Propionibacterium*, and *Atopobium* have been added to the list of candidate pathogens associated with this disease (14, 15, 18, 19). Moreover, studies of the microbiota of advanced dentinal caries reveal a predominance of lactobacilli and/or species/phylotypes of the genera *Prevotella*, *Selenomonas*, *Dialister*, *Fusobacterium*, *Eubacterium*, *Olsenella*, *Bifidobacterium*, members of the Lachnospiraceae family, and *Pseudoramibacter alactolyticus* (20–22).

Halitosis. Colonization of the tongue dorsum by bacteria producing volatile sulfur compounds and other metabolites has been implicated as a major source of oral malodor in subjects with halitosis (23). A molecular study revealed that about 60% of the bacteria detected on the tongue dorsum remains uncultivated and species-level taxa most associated with halitosis include *Atopobium parvulum*, *Eubacterium sulci*, *Solobacterium moorei*, and some as-yet-uncultivated phylotypes (*Dialister* clone BS095, TM7 clone DR034, and *Streptococcus* clone BW009) (5).

Periodontal and endodontic diseases. Periodontal diseases result from the subgingival presence of complex bacterial biofilms and endodontic diseases are caused by bacterial biofilms infecting the necrotic dental root canal. No specific etiologic agents have been unequivocally identified for both diseases (24, 25). Important advances in understanding the infectious agents of periodontal and endodontic diseases have occurred after introduction of molecular identification approaches. In addition to confirming the involvement of some anaerobic cultivable species, nucleic acid technology has also enabled identification of new bacterial species or phylotypes possibly implicated in the etiology of these diseases (7, 26–35). Bacteria commonly found in both periodontal and endodontic diseases belong to the following genera: *Porphyromonas* (e.g., *P. gingivalis* and *P. endodontalis*), *Prevotella* (e.g., *P. intermedia*, *P. nigrescens*, *P. baroniae*), *Tannerella* (e.g., *T. forsythia*), *Treponema* (e.g., *T. denticola* and *T. socranskii*), *Fusobacterium* (e.g., *F. nucleatum*), *Dialister* (e.g., *D. pneumosintes* and *D. invisus*), *Filifactor* (e.g., *F. alocis*), *Parvimonas* (e.g., *P. micra*), *Eubacterium* (e.g., *E. nodatum*, *E. sulci*), and many others. *Aggregatibacter actinomycetemcomitans* has been associated with some forms of periodontal disease (36), but not with endodontic diseases (37).

Broad-range polymerase chain reaction (PCR) and clone library studies have revealed that 40–60% of the microbiota associated with periodontal and endodontic diseases is made up of as-yet-uncultivated species-level phylotypes (7, 29, 33–35). Many of

these phylotypes have been frequently found in association with disease (7, 28, 29, 33, 34). Knowledge of the infectious etiologic agents of periodontal diseases keeps expanding as microorganisms other than bacteria, namely archaea (38, 39) and herpesviruses (40, 41), have also been found in association with periodontal and endodontic diseases.

As one can tell, a significant revolution in the knowledge of the oral microbiota in health and disease has taken place over the very recent years after the advent of nucleic acid technology for microbial identification. Table 5.1 depicts a taxonomic overview of the most common phyla and respective genera that have oral representatives.

Table 5.1
Bacterial phyla and respective genera commonly found in the oral cavity

Phyla and genera	Species-level representatives
Firmicutes	
<i>Anaerococcus</i>	<i>A. prevotii</i>
<i>Catonella</i>	<i>C. morbi</i>
<i>Centipeda</i>	<i>C. periodontii</i>
<i>Dialister</i>	<i>D. invisus</i> , <i>D. pneumosintes</i> , uncultivated phylotypes
<i>Eggerthella</i>	<i>E. lenta</i>
<i>Enterococcus</i>	<i>E. faecalis</i>
<i>Eubacterium</i>	<i>E. sulci</i> , <i>E. infirmum</i> , <i>E. saphenum</i> , <i>E. nodatum</i> , <i>E. brachy</i> , <i>E. minutum</i> , uncultivated phylotypes
<i>Filifactor</i>	<i>F. alocis</i>
<i>Finegoldia</i>	<i>F. magna</i>
<i>Gemella</i>	<i>G. morbillorum</i>
<i>Granulicatella</i>	<i>G. adiacens</i>
<i>Lactobacillus</i>	<i>L. salivarius</i> , <i>L. acidophilus</i> , <i>L. fermentum</i> , <i>L. paracasei</i> , <i>L. cateniformis</i>
<i>Megasphaera</i>	Uncultivated phylotypes
<i>Mogibacterium</i>	<i>M. timidum</i> , <i>M. pumilum</i> , <i>M. neglectum</i> , <i>M. vesicum</i>
<i>Parvimonas</i>	<i>P. micra</i>
<i>Peptoniphilus</i>	<i>P. asaccharolyticus</i> , <i>P. lacrimalis</i>
<i>Peptostreptococcus</i>	<i>P. anaerobius</i> , uncultivated phylotypes
<i>Pseudoramibacter</i>	<i>P. alactolyticus</i>
<i>Selenomonas</i>	<i>S. sputigena</i> , <i>S. noxia</i> , uncultivated phylotypes
<i>Solobacterium</i>	<i>S. moorei</i> , uncultivated phylotypes
<i>Streptococcus</i>	<i>S. mutans</i> , <i>S. sobrinus</i> , <i>S. mitis</i> , <i>S. sanguinis</i> , <i>S. gordonii</i> , <i>S. oralis</i> , <i>S. anginosus</i> , <i>S. constellatus</i> , <i>S. intermedius</i> , uncultivated phylotypes
<i>Veillonella</i>	<i>V. parvula</i> , uncultivated phylotypes

Table 5.1
(continued)

Phyla and genera	Species-level representatives
Bacteroidetes	
<i>Capnocytophaga</i>	<i>C. gingivalis</i> , <i>C. ochracea</i>
<i>Porphyromonas</i>	<i>P. endodontalis</i> , <i>P. gingivalis</i>
<i>Prevotella</i>	<i>P. intermedia</i> , <i>P. nigrescens</i> , <i>P. tannerae</i> , <i>P. multissacharivorax</i> , <i>P. baroniae</i> , <i>P. denticola</i> , uncultivated phylotypes
<i>Tannerella</i>	<i>T. forsythia</i>
Actinobacteria	
<i>Actinomyces</i>	<i>A. israelii</i> , <i>A. gerencseriae</i> , <i>A. naeslundii</i> , <i>A. meyeri</i> , <i>A. odontolyticus</i> , uncultivated phylotypes
<i>Atopobium</i>	<i>A. parvulum</i> , <i>A. minutum</i> , <i>A. rimae</i> , uncultivated phylotypes
<i>Bifidobacterium</i>	<i>B. dentium</i> , <i>B. adolescentis</i> , <i>B. bifidum</i>
<i>Corynebacterium</i>	<i>C. matruchotii</i>
<i>Olsenella</i>	<i>O. uli</i> , <i>O. profusa</i> , uncultivated phylotypes
<i>Propionibacterium</i>	<i>P. acnes</i> , <i>P. propionicum</i>
<i>Rothia</i>	<i>R. dentocariosa</i>
<i>Slackia</i>	<i>S. exigua</i>
Proteobacteria	
<i>Aggregatibacter</i>	<i>A. actinomycetemcomitans</i> , <i>A. aphrophilus</i>
<i>Campylobacter</i>	<i>C. rectus</i> , <i>C. gracilis</i> , <i>C. curvus</i> , <i>C. showae</i> , <i>C. concisus</i>
<i>Eikenella</i>	<i>E. corrodens</i>
<i>Neisseria</i>	<i>N. mucosa</i> , <i>N. sicca</i>
Fusobacteria	
<i>Fusobacterium</i>	<i>F. nucleatum</i> , <i>F. periodonticum</i> , uncultivated phylotypes
<i>Leptotrichia</i>	<i>L. buccalis</i>
Spirochaetes	
<i>Treponema</i>	<i>T. denticola</i> , <i>T. socranskii</i> , <i>T. parvum</i> , <i>T. maltophilum</i> , <i>T. lecithinolyticum</i> , uncultivated phylotypes

4. Nucleic Acid Techniques

Nucleic acid (or molecular biology) techniques have revolutionized the field of medical microbiology given its numerous advantages over other commonly used methods (**Table 5.2**). As with any other technology, molecular methods have also their own limitations, which are displayed in **Table 5.2**. A large selection of molecular methods for the study of microorganisms is currently available and the choice of a particular approach depends on the questions being addressed. As for the identification of

Table 5.2
Advantages and limitations of molecular techniques

Advantages	Limitations
1. Detect both cultivable and as-yet-uncultivated species or strains 2. High specificity and accurate identification of strains with ambiguous phenotypic behavior 3. Detect species directly in clinical samples 4. High sensitivity 5. Rapid – identification can be achieved in no more than minutes to a few hours 6. Do not require carefully controlled anaerobic conditions during sampling, transportation, and handling 7. Can be used during antimicrobial treatment 8. Samples can be stored frozen for later analysis 9. DNA can be transported easily between laboratories 10. Detect dead microorganisms^a	1. Most assays are qualitative or semi-quantitative (exceptions: real-time PCR, DNA microarrays) 2. Most assays only detect one species or a few different species at a time (exceptions: broad-range PCR, DGGE, T-RFLP, checkerboard, DNA microarrays, metagenomics) 3. Most assays detect only the target species and fail to detect unexpected species (exceptions: broad-range PCR, DGGE, T-RFLP, metagenomics) 4. Some assays can be laborious and costly (e.g., broad-range PCR, metagenomics) 5. Biases in broad-range PCR introduced by homogenization procedures, preferential DNA amplification, and differential DNA extraction 6. Hybridization assays using whole-genome probes detect only cultivable species 7. Detect dead microorganisms^a

^aThe detection of dead cells can be an advantage as well as a limitation. On the plus side, this ability allows detection of hitherto uncultivated or fastidious bacteria that can die during sampling, transportation, or isolation procedures. On the down side, detection of dead bacteria may give rise to misinterpretations as to their role in the habitat.

microbial species, molecular methods can be directly used in clinical samples to detect the unexpected (open-ended analysis) or to target specific taxa (closed-ended analysis). Broad-range PCR followed by cloning and sequencing (clone library analysis) can be used to disclose the microbial diversity in a given environment. Microbial community structures can be analyzed and components can be identified via community profiling techniques, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (described in **Chapter 6** by Siqueira et al., this volume). Among other applications, DNA–DNA hybridization arrays, specific single PCR, nested PCR, multiplex PCR, and quantitative real-time PCR can be used to survey large numbers of clinical samples for the presence of target species. Fluorescence in situ hybridization (FISH) can identify, measure abundance of target species, and provide information on their spatial distribution in tissues.

Molecular approaches for bacterial identification rely on certain genes that contain revealing information about the microbial identity. Ideally, a gene to be used as a target for bacterial identification should contain regions that are unique to each species.

Genes encoding housekeeping functions are preferable to infer phylogenetic classification since they are usually ubiquitous and tend to exhibit functional constancy, evolving slowly with time (42, 43).

Although several genes have been chosen as targets for bacterial identification (44, 45), the gene encoding the 16S rRNA has been widely accepted and used. The advantages of using the 16S rRNA genes for bacterial identification are that it is found in all bacteria, is long enough to be highly informative and short enough to be easily sequenced, and affords reliability for inferring phylogenetic relationships (46). In addition, there are many available public depositories for 16S rRNA gene sequences, which generate massive databases, e.g., the Ribosomal Database Project (RDP) for more precise species/phylotype identification and other purposes, such as probe and primer design.

The following is an overview of the most commonly used approaches applied to the research of the oral microbiota in health and disease.

5. PCR

The PCR method is based on the in vitro replication of DNA through repetitive cycles of denaturation, primer annealing, and extension steps carried out in automated devices (thermocyclers). The result is an exponential amplification of the genomic region flanked by the primers, which confers the extraordinary sensitivity of PCR in detecting the target DNA (47).

Numerous derivatives of the conventional PCR technology have been developed. The most used PCR-derived assays in oral microbiology research are described below.

Species-specific PCR. This is one of the simplest approaches to detect a target species in a sample. By this method, primers designed to anneal to signature genomic sequences of a given species are used to detect this species directly in clinical samples even against a background of nontargeted species and without the need for cultivation. The presence of a species-specific PCR product of predicted size is usually determined by agarose gel electrophoresis and represents a positive result for the occurrence of the target species in the sample. Sequencing of the PCR product should be performed to confirm method's specificity. This approach can be used not only in single PCR assays, but also in nested PCR and multiplex PCR, furnishing qualitative results (presence or absence) about one (the two former techniques) or more (the latter technique) target species. Species-specific

detection can also be performed using a quantitative real-time PCR assay, which detects and monitors the appearance of the amplification product throughout the reaction.

Nested PCR. Nested PCR consists of two rounds of amplification using different sets of primers in each round. A target region of DNA is amplified with an outer primer pair in an initial reaction, followed by a second amplification using an internal primer pair. Primers used in the second round of amplification can be different to those in the first set (nested) or that one of the primers can be common to both sets (hemi- or semi-nested). This approach has been devised mainly to have increased sensitivity (48), but can also exhibit increased specificity (47).

Multiplex PCR. In multiplex PCR, two or more sets of primers specific for different targets that generate amplicons of different sizes are concomitantly used in the same reaction (49). This allows for the simultaneous detection of different species in a sample.

Reverse transcriptase PCR (RT-PCR). RT-PCR was developed to amplify RNA targets and exploits the use of the enzyme reverse transcriptase, which can synthesize a strand of complementary DNA (cDNA) from an RNA template.

Quantitative real-time PCR. PCR assays are usually qualitative or can be adjusted to be semi-quantitative. One exception is real-time PCR, in which a fluorescent indicator dye is used in the reaction to allow quantification of the amount of DNA in the sample by monitoring, in real time, the release of fluorescence during each amplification cycle. The fluorescent signal is proportional to the amount of DNA synthesis and is measured automatically during each cycle in a closed tube format using a thermocycler combined with a fluorimeter. Real-time PCR assays allow the quantification of individual target species as well as total bacteria in clinical samples. There are several different real-time PCR approaches, but the most commonly used chemistries include SYBRTM-Green (50) and TaqMan (51).

Broad-range PCR. PCR technology can be used to investigate the breadth of microbial diversity in a given environment. In broad-range PCR, primers are designed that are complementary to conserved regions of a particular gene shared by a group of microorganisms. For instance, primers that are complementary to conserved regions of the 16S rRNA gene have been used with the intention of exploiting the variable internal regions of the amplified sequence for sequencing and further identification (52). Initially, bacterial DNA is extracted directly from samples and the 16S rRNA gene is isolated via PCR amplification with oligonucleotide primers specific for conserved regions of the gene (universal or broad-range primers). Amplification with universal primers results in a mixture of the 16S rRNA genes amplified from virtually all bacteria present in the sample. In mixed infections,

direct sequencing of the PCR products cannot be performed because there are mixed products from the different species composing the consortium. PCR products are then cloned into a plasmid vector, which is used to transform *Escherichia coli* cells, establishing a clone library of 16S rRNA gene from the sample. Cloned genes are then sequenced individually and identification is achieved by performing similarity searches in public databases and further phylogenetic analysis (53, 54). Broad-range PCR and clone library analysis have allowed the identification of several novel fastidious or as-yet-uncultivated bacterial pathogens directly from diverse human oral sites (4, 8, 14, 16, 18, 29, 33–35).

Broad-range PCR products from samples can be alternatively analyzed by fingerprinting techniques, such as DGGE and T-RFLP. Genetic fingerprinting techniques can be used to profile microbial communities living in a given environment and to monitor changes over time. DGGE and T-RFLP are also referred to as community profiling techniques (*see Chapter 6* by Siqueira et al., this volume).

6. DNA–DNA Hybridization

DNA–DNA hybridization methodology is the process of annealing the complementary bases of two single-stranded DNA molecules. It employs labeled single-stranded DNA probes that can locate and bind to a target sequence, forming a new duplex molecule. The labeled duplex can then be detected (55). Probes are constructed from either whole genomic DNA or oligonucleotides. Whole genomic probes are more likely to cross-react with nontarget microorganisms due to the presence of homologous sequences between different species. Oligonucleotide probes based on signature sequences of specific genes (such as the 16S rRNA gene) may display limited or no cross-reactivity with nontarget microorganisms when under optimized conditions. In addition, oligonucleotide probes can differentiate between closely related species or even subspecies and can be designed to detect as-yet-uncultivated bacteria. Hybridization methods developed for large-scale studies include the checkerboard DNA–DNA hybridization and DNA microarray techniques.

Checkerboard DNA–DNA hybridization. This technique was introduced by Socransky et al. (56) for hybridizing large numbers of DNA samples against large numbers of digoxigenin-labeled whole genomic DNA or 16S rRNA gene-based oligonucleotide probes on a single support membrane. Briefly, denatured DNA from clinical samples is placed in lanes on a nylon membrane using

a Minislot apparatus. After fixation of the samples to the membrane, the membrane is placed in a Miniblotter 45 apparatus with the lanes of samples at 90° to the lanes of the device. Digoxigenin-labeled whole genomic DNA probes are then loaded in individual lanes of the Miniblotter. After hybridization, the membranes are washed at high stringency and the DNA probes detected using antibody to digoxigenin conjugated with alkaline phosphatase and chemifluorescence or chemiluminescence detection. The checkerboard method permits the simultaneous determination of the presence of a multitude of bacterial species in single or multiple clinical samples. A modification of the checkerboard method was proposed by Paster et al. (57) and consists of a PCR-based, reverse-capture checkerboard hybridization methodology. The procedure circumvents the need for bacterial culture, a necessary step in preparing whole genomic probes. Up to 30 reverse-capture oligonucleotide probes that target regions of the 16S rRNA gene are deposited on a nylon membrane in separate horizontal lanes using a Minislot apparatus. Probes are synthesized with a polythymidine tail, which are cross-linked to the membrane via ultraviolet irradiation or heat, leaving the probes available for hybridization. The 16S rRNA gene from clinical samples is PCR amplified using a digoxigenin-labeled primer. Hybridizations are performed in vertical channels in a Miniblotter apparatus with digoxigenin-labeled PCR amplicons for up to 45 samples. Hybridization signals are detected using chemifluorescence or chemiluminescence procedures. The reverse-capture checkerboard assay has important advantages over the original checkerboard method, mostly related to the use of oligonucleotide probes instead of whole genomic probes (57). Whereas oligonucleotide probes display higher specificity and can be designed to detect both cultivable and as-yet-uncultivated bacteria, the original checkerboard method employing whole genomic probes detects only those cultivable species that are targeted (58). Nevertheless, the trade-off for the ability to detect any species of interest is the loss of quantitative assessment due to biases related to the PCR amplification step (59).

DNA microarrays. DNA microarrays consist of a high-density matrix of DNA probes which are printed or synthesized on a glass or silicon slide (chip) (60). Labeled DNA targets are applied to the array and those that hybridize to complementary probes are detected using some type of reporter molecule. Following hybridization, arrays are imaged using a high-resolution scanner and analyzed by sophisticated computer software programs. PCR can be used to amplify microbial DNA from clinical specimens and then microarrays are used to identify the PCR products by hybridization to an array that is composed of species-specific probes (10, 61). Using broad-range primers, such as those that amplify the 16S rRNA gene, a single PCR can be used to

detect hundreds to thousands of bacterial species simultaneously (10, 61).

Fluorescence in situ hybridization (FISH). This method uses fluorescently labeled oligonucleotide probes in combination with fluorescence microscopy to detect intact bacterial cells directly in clinical specimens (62). In addition to facilitating species identification, FISH provides information about presence, morphology, number, organization, and spatial distribution of bacteria (63). Because a variety of oligonucleotide probes can be designed, FISH allows not only the detection of cultivable microbial species, but also of as-yet-uncultivated microorganisms (64, 65). A typical FISH protocol includes four steps: (i) fixation and permeabilization of the sample, (ii) hybridization with the respective probes for detecting the respective target sequences, (iii) washing steps to remove unbound probe, and (iv) detection of labeled cells by microscopy or flow cytometry (66).

7. Metagenomics

Metagenomics is the culture-independent analysis of the collective microbial genomes (termed the metagenome) in an environmental community, using a PCR-independent approach (67). Metagenomics treats the genomes of all microorganisms present in a specific habitat as an entity. Theoretically, a metagenomic library will contain DNA sequences for all the genes in the microbial community. Metagenomics provides a comprehensive view not only of the community structure (richness and distribution of species) but also of the functional potential of a community (68).

The metagenomic approach typically begins with the construction of a clone library from DNA retrieved from environmental or clinical samples. Extracted DNA is cloned into large insert cloning vectors, such as fosmids or bacterial artificial chromosomes (BACs). BACs have the advantage that they can be used to maintain and express the insert genes in the host harboring the vector (69). *E. coli* is the preferred host for the cloning and expression of metagenome-derived genes. Clones are then selected for screening using either functional or sequence-based approaches. In the functional approach, genes retrieved from the environment are heterologously expressed in a host, such as *E. coli*, and sophisticated functional screens are employed to detect clones expressing functions of interest. In the sequence-based approach, clones are selected for sequencing based on the presence of either phylogenetically informative genes, such as the 16S rRNA gene, or other genes of interest (70). Facilitated by the increasing capacity of sequencing centers, whole-genome

shotgun sequencing of the entire clone library has emerged as a third approach to metagenomics. Unlike previous approaches, which typically study a single gene or individual genomes, this approach offers a more global view of the community, allowing the better assessment of levels of phylogenetic diversity and intraspecies polymorphism, study of the metabolic pathways in the community, and, in some cases, reconstruction of the near-complete genome sequences (71). Shotgun sequencing also has the potential to disclose new genes that are too different from known genes to be amplified with PCR or heterologously expressed in common hosts (70, 71).

Metagenomic analysis of the oral microbiome holds the potential to provide invaluable information about the physiological and functional roles of the oral microbiota, including bacteria that have not yet been cultivated.

8. Concluding Remarks

Traditionally, the oral microbiota in health and disease has been studied by means of culture approaches. Such studies have resulted in the establishment of a set of species thought to play an important role in the pathogenesis of several oral diseases. More recently, not only have findings from culture-based methods been confirmed but they have also been significantly supplemented with those from culture-independent nucleic acid techniques. Molecular methods have confirmed and strengthened the association of many cultivable bacterial species with oral diseases and have also revealed new suspected pathogens. The list of oral inhabitants, including candidate pathogens, has expanded to include culture-difficult species or even as-yet-uncultivated bacteria that had never been previously found by culturing approaches. As a consequence of the resolution and high throughput of many molecular biology approaches, the oral microbiota has been comprehensively refined.

References

1. Leys, E. J., Griffen, A. L., Kumar, P. S., and Maiden, M. F. (2006) Isolation, classification, and identification of oral microorganisms, in *Oral microbiology and immunology* (Lamont, R. J., Burne, R. A., Lantz, M. S., and Leblanc, D. J., Eds.). ASM Press, Washington, DC, pp. 73–88.
2. Socransky, S. S., Gibbons, R. J., Dale, A. C., Bortnick, L., Rosenthal, E., and MacDonald, J. B. (1963) The microbiota of the gingival crevice in man. 1. Total microscopic and viable counts and counts of specific organisms. *Arch. Oral. Biol.* **8**, 275–280.
3. Paster, B. J., Olsen, I., Aas, J. A., and Dewhirst, F. E. (2006) The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol 2000*. **42**, 80–87.

4. Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., and Dewhirst, F. E. (2005) Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* **43**, 5721–5732.
5. Kazor, C. E., Mitchell, P. M., Lee, A. M., Stokes, L. N., Loesche, W. J., Dewhirst, F. E., and Paster, B. J. (2003) Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *J. Clin. Microbiol.* **41**, 558–563.
6. Paster, B. J., Falkler, W. A., Jr, Enwonwu, C. O., Idigbe, E. O., Savage, K. O., Levanos, V. A., Tamer, M. A., Ericson, R. L., Lau, C. N., and Dewhirst, F. E. (2002) Prevalent bacterial species and novel phylotypes in advanced noma lesions. *J. Clin. Microbiol.* **40**, 2187–2191.
7. Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A., and Dewhirst, F. E. (2001) Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **183**, 3770–3783.
8. Lillo, A., Ashley, F. P., Palmer, R. M., Munson, M. A., Kyriacou, L., Weightman, A. J., and Wade, W. G. (2006) Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets. *Oral Microbiol. Immunol.* **21**, 61–68.
9. Aas, J. A., Barbutto, S. M., Alpagot, T., Olsen, I., Dewhirst, F. E., and Paster, B. J. (2007) Subgingival plaque microbiota in HIV positive patients. *J. Clin. Periodontol.* **34**, 189–195.
10. Huyghe, A., Francois, P., Charbonnier, Y., Tangomo-Bento, M., Bonetti, E. J., Paster, B. J., Bolivar, I., Baratti-Mayer, D., Pittet, D., and Schrenzel, J. (2008) Novel microarray design strategy to study complex bacterial communities. *Appl. Environ. Microbiol.* **74**, 1876–1885.
11. Keijser, B. J., Zaura, E., Huse, S. M., van der Vossen, J. M., Schuren, F. H., Montijn, R. C., Ten Cate, J. M., and Crielaard, W. (2008) Pyrosequencing analysis of the oral microflora of healthy adults. *J. Dent. Res.* **87**, 1016–1020.
12. Marsh, P., and Martin, M. V. (1999) *Oral microbiology*, 4th ed. Wright, Oxford.
13. Bowden, G. H. (2000) The microbial ecology of dental caries. *Microb. Ecol. Health Dis.* **12**, 138–148.
14. Preza, D., Olsen, I., Aas, J. A., Willumsen, T., Grinde, B., and Paster, B. J. (2008) Bacterial profiles of root caries in elderly patients. *J. Clin. Microbiol.* **46**, 2015–2021.
15. Aas, J. A., Dardis, S. R., Griffen, A. L., Stokes, L. N., Lee, A. M., Olsen, I., Dewhirst, F. E., Leys, E. J., and Paster, B. J. (2003) Molecular analysis of bacteria associated with caries in permanent teeth. *J. Dent. Res.* **82**(Spec Iss A), IADR Abstract No. 1025. <http://www.dentalresearch.org>.
16. Munson, M. A., Banerjee, A., Watson, T. F., and Wade, W. G. (2004) Molecular analysis of the microflora associated with dental caries. *J. Clin. Microbiol.* **42**, 3023–3029.
17. Aas, J. A., Dardis, S. R., Griffen, A. L., Stokes, L. N., Lee, A. M., Olsen, I., Dewhirst, F. E., Leys, E. J., and Paster, B. J. (2005) Most of the microbiota in caries has not yet been cultivated. *J. Dent. Res.* **84**(Spec Iss A), IADR Abstract No 2805. <http://www.dentalresearch.org>.
18. Aas, J. A., Griffen, A. L., Dardis, S. R., Lee, A. M., Olsen, I., Dewhirst, F. E., Leys, E. J., and Paster, B. J. (2008) Bacteria of dental caries in primary and permanent teeth in children and young adults. *J. Clin. Microbiol.* **46**, 1407–1417.
19. Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., Boches, S. K., Dewhirst, F. E., and Griffen, A. L. (2002) Molecular analysis of bacterial species associated with childhood caries. *J. Clin. Microbiol.* **40**, 1001–1009.
20. Nadkarni, M. A., Caldon, C. E., Chhour, K. -L., Fisher, I. P., Martin, F. E., Jacques, N. A., and Hunter, N. (2004) Carious dentine provides a habitat for a complex array of novel *Prevotella*-like bacteria. *J. Clin. Microbiol.* **42**, 5238–5244.
21. Martin, F. E., Nadkarni, M. A., Jacques, N. A., and Hunter, N. (2002) Quantitative microbiological study of human carious dentine by culture and real-time PCR: association of anaerobes with histopathological changes in chronic pulpitis. *J. Clin. Microbiol.* **40**, 1698–1704.
22. Chhour, K. L., Nadkarni, M. A., Byun, R., Martin, F. E., Jacques, N. A., and Hunter, N. (2005) Molecular analysis of microbial diversity in advanced caries. *J. Clin. Microbiol.* **43**, 843–849.
23. Loesche, W. J., and Kazor, C. (2002) Microbiology and treatment of halitosis. *Periodontol 2000*. **28**, 256–279.
24. Siqueira, J. F., Jr. (2002) Endodontic infections: concepts, paradigms, and perspectives. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **94**, 281–293.
25. Moore, W. E. C., and Moore, L. V. H. (1994) The bacteria of periodontal diseases. *Periodontol 2000*. **5**, 66–77.
26. Sakamoto, M., Huang, Y., Umeda, M., Ishikawa, I., and Benno, Y. (2002) Detection of novel oral phylotypes associated with periodontitis. *FEMS Microbiol. Lett.* **217**, 65–69.

27. Griffen, A. L., Kumar, P. S., and Leys, E. J. (2003) *A quantitative, molecular view of oral biofilm communities in health and disease suggests a role for uncultivated species, Polymicrobial diseases*. American Society for Microbiology conferences, Lake Tahoe, p. 13.
28. Kumar, P. S., Griffen, A. L., Barton, J. A., Paster, B. J., Moeschberger, M. L., and Leys, E. J. (2003) New bacterial species associated with chronic periodontitis. *J. Dent. Res.* **82**, 338–344.
29. Kumar, P. S., Griffen, A. L., Moeschberger, M. L., and Leys, E. J. (2005) Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. *J. Clin. Microbiol.* **43**, 3944–3955.
30. Brinig, M. M., Lepp, P. W., Ouverney, C. C., Armitage, G. C., and Relman, D. A. (2003) Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. *Appl. Environ. Microbiol.* **69**, 1687–1694.
31. Harper-Owen, R., Dymock, D., Booth, V., Weightman, A. J., and Wade, W. G. (1999) Detection of unculturable bacteria in periodontal health and disease by PCR. *J. Clin. Microbiol.* **37**, 1469–1473.
32. Siqueira, J. F., Jr, and Rôças, I. N. (2005) Exploiting molecular methods to explore endodontic infections: Part 2 – redefining the endodontic microbiota. *J. Endod.* **31**, 488–498.
33. Sakamoto, M., Siqueira, J. F., Jr, Rôças, I. N., and Benno, Y. (2008) Molecular analysis of the root canal microbiota associated with endodontic treatment failures. *Oral Microbiol. Immunol.* **23**, 275–281.
34. Sakamoto, M., Rôças, I. N., Siqueira, J. F., Jr, and Benno, Y. (2006) Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. *Oral Microbiol. Immunol.* **21**, 112–122.
35. Munson, M. A., Pitt-Ford, T., Chong, B., Weightman, A., and Wade, W. G. (2002) Molecular and cultural analysis of the microflora associated with endodontic infections. *J. Dent. Res.* **81**, 761–766.
36. Slots, J., and Ting, M. (1999) Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in human periodontal disease: occurrence and treatment. *Periodontol 2000*. **20**, 82–121.
37. Siqueira, J. F., Jr, Rôças, I. N., Moraes, S. R., and Santos, K. R. (2002) Direct amplification of rRNA gene sequences for identification of selected oral pathogens in root canal infections. *Int. Endod. J.* **35**, 345–351.
38. Lepp, P. W., Brinig, M. M., Ouverney, C. C., Palm, K., Armitage, G. C., and Relman, D. A. (2004) Methanogenic Archaea and human periodontal disease. *Proc. Natl. Acad. Sci. USA*. **101**, 6176–6181.
39. Vianna, M. E., Conrads, G., Gomes, B. P. F. A., and Horz, H. P. (2006) Identification and quantification of archaea involved in primary endodontic infections. *J. Clin. Microbiol.* **44**, 1274–1282.
40. Sabeti, M., Simon, J. H., and Slots, J. (2003) Cytomegalovirus and Epstein-Barr virus are associated with symptomatic periapical pathosis. *Oral Microbiol. Immunol.* **18**, 327–328.
41. Slots, J. (2005) Herpesviruses in periodontal diseases. *Periodontol 2000*. **38**, 33–62.
42. Wade, W. G. (2004) Non-culturable bacteria in complex commensal populations. *Adv. Appl. Microbiol.* **54**, 93–106.
43. Woese, C. R. (2000) Interpreting the universal phylogenetic tree. *Proc. Natl. Acad. Sci. USA*. **97**, 8392–8396.
44. Drancourt, M., and Raoult, D. (2005) Sequence-based identification of new bacteria: a proposition for creation of an orphan bacterium repository. *J. Clin. Microbiol.* **43**, 4311–4315.
45. Ke, D., Picard, F. J., Martineau, F., Menard, C., Roy, P. H., Ouellette, M., and Bergeron, M. G. (1999) Development of a PCR assay for rapid detection of enterococci. *J. Clin. Microbiol.* **37**, 3497–3503.
46. Woese, C. R. (1987) Bacterial evolution. *Microbiol. Rev.* **51**, 221–271.
47. Siqueira, J. F., Jr, and Rôças, I. N. (2003) PCR methodology as a valuable tool for identification of endodontic pathogens. *J. Dent.* **31**, 333–339.
48. Haqqi, T. M., Sarkar, G., David, C. S., and Sommer, S. S. (1988) Specific amplification with PCR of a refractory segment of genomic DNA. *Nucleic Acids Res.* **16**, 11844.
49. Chamberlain, J. S., Gibbs, R. A., Ranier, J. E., Nguyen, P. N., and Caskey, C. T. (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res.* **16**, 11141–11156.
50. Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. (1992) Simultaneous amplification and detection of specific DNA sequences. *Biotechnology (NY)*. **10**, 413–417.
51. Heid, C. A., Stevens, J., Livak, K. J., and Williams, P. M. (1996) Real time quantitative PCR. *Genome Res.* **6**, 986–994.
52. Göbel, U. B. (1995) Phylogenetic amplification for the detection of uncultured

- bacteria and the analysis of complex microbiota. *J. Microbiol. Methods*. **23**, 117–128.
53. Lepp, P. W., and Relman, D. A. (2004) Molecular phylogenetic analysis, in *Molecular microbiology. Diagnostic principles and practice* (Persing, D. H., Tenover, F. C., Versalovic, J., Tang, Y.-W., Unger, E. R., Relman, D., and White, T. J., Eds.). ASM Press, Washington, DC, pp. 161–180.
54. Maiwald, M. (2004) Broad-range PCR for detection and identification of bacteria. in *Molecular microbiology. Diagnostic principles and practice* (Persing, D. H., Tenover, F. C., Versalovic, J., Tang, Y.-W., Relman, D., and White, T. J., Eds.). ASM Press, Washington, DC, pp. 379–390.
55. Li, J., and Hanna, B. A. (2004) DNA probes for culture confirmation and direct detection of bacterial infections: a review of technology, in *Molecular microbiology. Diagnostic principles and practice* (Persing, D. H., Tenover, F. C., Versalovic, J., Tang, Y.-W., Unger, E. R., Relman, D. A., and White, T. J., Eds.). ASM Press, Washington, DC, pp. 19–26.
56. Socransky, S. S., Smith, C., Martin, L., Paster, B. J., Dewhirst, F. E., and Levin, A. E. (1994) “Checkerboard” DNA–DNA hybridization. *Biotechniques*. **17**, 788–792.
57. Paster, B. J., Bartoszyk, I. M., and Dewhirst, F. E. (1998) Identification of oral streptococci using PCR-based, reverse-capture, checkerboard hybridization. *Methods Cell Sci.* **20**, 223–231.
58. Rôças, I. N., and Siqueira, J. F., Jr. (2008) Root canal microbiota of teeth with chronic apical periodontitis. *J. Clin. Microbiol.* **46**, 3599–3606.
59. Kuramitsu, H. K., He, X., Lux, R., Anderson, M. H., and Shi, W. (2007) Interspecies interactions within oral microbial communities. *Microbiol. Mol. Biol. Rev.* **71**, 653–670.
60. Mothershed, E. A., and Whitney, A. M. (2006) Nucleic acid-based methods for the detection of bacterial pathogens: present and future considerations for the clinical laboratory. *Clin. Chim. Acta.* **363**, 206–220.
61. Palmer, C., Bik, E. M., Eisen, M. B., Eckburg, P. B., Sana, T. R., Wolber, P. K., Relman, D. A., and Brown, P. O. (2006) Rapid quantitative profiling of complex microbial populations. *Nucleic Acids Res.* **34**, e5.
62. Moter, A., and Gobel, U. B. (2000) Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. *J. Microbiol. Methods*. **41**, 85–112.
63. Amann, R., Fuchs, B. M., and Behrens, S. (2001) The identification of microorganisms by fluorescence in situ hybridisation. *Curr. Opin. Biotechnol.* **12**, 231–236.
64. Moter, A., Leist, G., Rudolph, R., Schrank, K., Choi, B. K., Wagner, M., and Gobel, U. B. (1998) Fluorescence in situ hybridization shows spatial distribution of as yet uncultured treponemes in biopsies from digital dermatitis lesions. *Microbiology*. **144**, 2459–2467.
65. Moter, A., Hoenig, C., Choi, B. K., Riep, B., and Gobel, U. B. (1998) Molecular epidemiology of oral treponemes associated with periodontal disease. *J. Clin. Microbiol.* **36**, 1399–1403.
66. Wagner, M., Horn, M., and Daims, H. (2003) Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. *Curr. Opin. Microbiol.* **6**, 302–309.
67. Handelsman, J. (2004) Metagenomics: application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. Rev.* **68**, 669–685.
68. Hugenholtz, P., and Tyson, G. W. (2008) Microbiology: metagenomics. *Nature*. **455**, 481–483.
69. Xu, J. (2006) Microbial ecology in the age of genomics and metagenomics: concepts, tools, and recent advances. *Mol. Ecol.* **15**, 1713–1731.
70. Hugenholtz, P. (2002) Exploring prokaryotic diversity in the genomic era. *Genome Biol.* **3**, reviews0003.1–0003.8.
71. Chen, K., and Pachter, L. (2005) Bioinformatics for whole-genome shotgun sequencing of microbial communities. *PLoS Comput. Biol.* **1**, 106–112.

Chapter 6

Microbial Community Profiling Using Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE)

José F. Siqueira Jr., Mitsuo Sakamoto, and Alexandre S. Rosado

Abstract

In their natural environments, microorganisms usually live in organized communities. Profiling analysis of microbial communities has recently assumed special relevance as it allows a thorough understanding of the diversity of the microbiota, its behavior over time, and the establishment of patterns associated with health and disease. The application of molecular biology approaches holds the advantage of including culture-difficult and as-yet-uncultivated phylotypes in the profiles, providing a more comprehensive picture of the microbial community. This chapter focuses on two particular techniques: the terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE), both of which have been widely used in environmental studies and have been recently successfully used by the authors in the study of the oral microbial communities associated with conditions of health and disease.

Key words: Human oral microbiota, 16S rRNA gene, terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE).

1. Introduction

Microbial community profiling techniques are genetic fingerprinting approaches that can be used to determine the structure and diversity of microbial communities living in a given environment and to monitor changes in the community over time, including after antimicrobial treatment. Species identification can also be obtained with these techniques. There are several molecular methods for community profiling, but the terminal restriction fragment length polymorphism (T-RFLP) and the denaturing

gradient gel electrophoresis (DGGE) have been frequently used in the study of oral communities in health and disease (1–12).

T-RFLP allows the assessment of the diversity of complex bacterial communities and rapid comparison of the community structure from different ecosystems (13). T-RFLP analysis measures the size polymorphism of terminal restriction fragments from a PCR-amplified marker. When T-RFLP is used to analyze bacterial communities, PCR is first carried out to amplify the 16S rRNA gene from different species in the sample. One of the PCR primers is labeled with a fluorescent dye (14). PCR amplicons are then digested with restriction enzymes, generating fluorescently labeled fragments of different lengths (the terminal fragments). These fragments are separated on high-resolution sequencing gels in an automated DNA sequencer, which is used to read both the size and the intensity of terminally labeled restriction fragments (T-RF), creating a typical profile. In such a profile, size is represented on the horizontal axis and intensity (relative to the abundance of a given fragment size) is represented on the vertical axis (15). In theory, each T-RF represents a single species. Extensive databases exist for 16S rRNA gene sequences and can be used to identify all T-RFs predicted from known sequences, considering a given set of primers and restriction enzymes (16). T-RF lengths are predicted by finding the restriction site closest to the site where the labeled primer will anneal and counting the number of nucleotides in between. Multiple restriction enzymes (usually four or five) are necessary to provide reliable identification since distinct species may generate the same T-RF when only one enzyme is used (17).

The DGGE technique is based on electrophoretic separation of PCR-amplified 16S rRNA gene (or other genes) fragments in polyacrylamide gels containing a linearly increasing gradient of DNA denaturants (a mixture of urea and formamide). As the PCR product migrates in the gel, it encounters increasing concentrations of denaturants and, at some position in the gel, it will become partially or fully denatured. Partial denaturation causes a significant decrease in the electrophoretic mobility of the DNA molecule. Molecules with different sequences may have a different melting behavior and will therefore stop migrating at different positions in the gel. The position in the gel at which the DNA melts is determined by its nucleotide sequence and composition (18). Therefore, in DGGE, PCR products of the same length but with different sequences can be separated (19, 20). A GC-rich sequence (or GC-clamp) is added to the 5'-end of one of the primers used in the PCR reaction and makes the DNA unable to denature completely in the gel (21). DNA bands in DGGE can be visualized using ethidium bromide, SYBRTM

Green, or silver staining. If species identification is desired, specific bands can be excised from the gels, re-amplified by PCR, and sequenced (22).

2. Materials

2.1. DNA Extraction

1. Buffer A: 10 mM Tris-HCl, pH 8.0, 50 mM ethylenediamine tetraacetic acid (EDTA).
2. Lysis buffer: 0.5% (w/v) lysozyme (Seikagaku Biobusiness Corporation, Tokyo, Japan) and 0.1% (w/v) *N*-acetylmuramidase (Seikagaku Biobusiness Corporation) in buffer A. Store in aliquots at -20°C .
3. TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
4. Alternatively, other techniques may be used for DNA extraction (*see* **Note 1**).

2.2. Terminal Restriction Fragment Length Polymorphism

2.2.1. PCR Amplification of the 16S rRNA Gene

1. Forward primer 8f: 5'-AGA GTT TGA TCC TGG CTC AG-3'. This primer is labeled at the 5'-end with 6'-carboxyfluorescein (6-FAM), which is synthesized by Applied Biosystems, Japan (*see* **Note 2**).
2. Reverse primer 1492r: 5'-GGT TAC CTT GTT ACG ACT T-3'.
3. Tris-acetate, EDTA (TAE) buffer (50 $\times$): 2 M Tris (do not adjust pH), 2 M glacial acetic acid, 0.05 M EDTA, pH 8.0.
4. Polyethyleneglycol (PEG) solution: 40% (w/v) PEG 6,000, 10 mM MgCl_2 (*see* **Note 3**).

2.2.2. T-RFLP Analysis

1. Capillaries: 310 Capillary 47 cm, 3130xl & 3100 Capillary Array 36 cm, 3130xl & 3100 Capillary Array 50 cm (Applied Biosystems, Foster City, CA, USA) (*see* **Note 4**).
2. Polymers: POP-4 (for ABI Genetic Analyzer 310 and ABI PRISM 3100 instruments); POP-7 (for ABI Genetic Analyzer model 3130xl) (Applied Biosystems).
3. Running buffer: Buffer (10 $\times$) with EDTA (Applied Biosystems).

4. Size standards: GeneScan 500 ROX Size Standard, Gene Scan 1000 ROX Size Standard, GeneScan 1200 LIZ Size Standard (all supplied by Applied Biosystems).
5. Template preparation reagent: Hi-Di Formamide (Applied Biosystems).

2.3. Denaturing Gradient Gel Electrophoresis

2.3.1. PCR Amplification of 16S rRNA Gene

1. Forward primer 968f: 5'-AAC GCG AAG AAC CTT AC-3', containing a 40-base GC-clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') added to its 5'-end, which makes it suitable for DGGE.
2. Reverse primer 1401r: 5'-GCG TGT GTA CAA GAC CC-3'.
3. Deionized formamide (*see* below).
4. Bovine serum albumin (BSA) 1% (w/v). Store in aliquots of 50 μ L at -20°C .

2.3.2. DGGE Analysis

1. TAE buffer: Tris/acetic acid/EDTA buffer 50 $\times$ (Bio-Rad 161-0743).
2. 1 $\times$ TAE buffer: (20 mM Tris-acetate (pH 7.4), 10 mM sodium acetate, 0.5 mM disodium EDTA). Store at room temperature.
3. Deionized formamide: add 12.5 g of AG 501-X8 resin (Bio-Rad, 142-6424) to 250 mL formamide 100% (Sigma, F-7503). Stir for 1 h at room temperature. Remove beads by passing the solution through folded filter paper in a funnel. Store in the dark at 4°C .
4. Ammonium persulfate (APS) (Bio-Rad, 161-0700): 10% (w/v) in deionized water. Store in 800 μ L aliquots at -20°C .
5. N, N, N, N'-tetramethylethylenediamine (TEMED) (Bio-Rad 161-0800).
6. Loading buffer 6 $\times$: 1.5 mL glycerol and 12.5 mg bromophenol blue (BPB) in 5 mL deionized water. Store at 4°C .
7. Gel-dye: 0.05 g bromophenol blue in 10 mL 1 $\times$ TAE.
8. Acrylamide/bis-acrylamide, 40% solution for electrophoresis, 37.5:1 (Sigma-Aldrich A7168).
9. Zero percentage UF (urea/formamide) in 6% acrylamide/bis: 15% (v/v) acrylamide/bis-acrylamide, 40% solution for electrophoresis (37.5:1), 2% (v/v) TAE buffer

50×. Store at 4°C in a dark bottle (storable up to 6 months) (*see* **Note 5**).

10. 100% UF in 6% acrylamide/bis: 42% (w/v) urea P.A., 40% (v/v) deionized formamide, 15% (v/v) acrylamide/bis-acrylamide, 40% solution for electrophoresis, 2% (v/v) TAE buffer 50×. The final volume must be completed to 100 mL after dissolving the urea (*see* **Note 6**).
11. Staining solution for DGGE: SYBRTM Green[®] in deionized (Milli-Q) water in the proportion of 1:10,000 (this solution should be prepared fresh and kept in the dark or in an amber vial).

3. Methods

3.1. DNA Extraction

1. An aliquot of 0.5 mL of clinical sample (saliva, pus, and plaque or root canal contents suspended in Tris-EDTA buffer) is diluted with buffer A in a 1:2 ratio (v/v) and washed with the same buffer (*see* **Note 1**).
2. The bacterial cell pellet obtained is resuspended in 0.5 mL of the lysis buffer. After incubation at 37°C for 1 h, proteinase K and sodium dodecyl sulfate (SDS) are added to a final concentration of 2 mg/mL and 1% (w/v), respectively. The mixture is incubated at 50°C for 2 h.
3. Nucleic acid is released by three cycles of freezing in a -80°C freezer followed by thawing in a 65°C water bath.
4. The nucleic acid is then extracted with equal volumes of phenol (saturated with 10 mM Tris-HCl, pH 8.0) and phenol:chloroform:isoamyl alcohol (25:24:1).
5. Bulk nucleic acids are precipitated from solution with 0.1 volume of 3 M sodium acetate and 0.8 volume of isopropyl alcohol followed by centrifugation (16,000*g* for 15 min).
6. The DNA precipitate is washed with 70% ethanol and resuspended in 100 µL TE.
7. RNase is added to a final concentration of 10 µg/mL and the mixture is incubated at 37°C for 1 h.
8. The mixture is then treated with equal volumes of phenol and phenol:chloroform:isoamyl alcohol (25:24:1).
9. The DNA is precipitated again with 0.1 volume of 3 M sodium acetate and 0.8 volume of isopropyl alcohol.
10. The DNA is pelleted by centrifugation (16,000*g* for 15 min), washed with 70% ethanol, dried in vacuum for 10 min, and dissolved in 100 µL TE.

3.2. Terminal Restriction Fragment Length Polymorphism

3.2.1. PCR Amplification of 16S rRNA Gene

1. Amplification reactions are performed in a total volume of 50 μL containing 5 μL of DNA extract (100 ng), 1.25 U Takara *Ex Taq* (Takara Bio, Japan), 5 μL of 10 $\times$ *Ex Taq* buffer, 4 μL of dNTP mixture (2.5 mM each), and 10 pmol of each primer.
2. 16S rRNA genes are amplified in a Biometra Tgradient Thermocycler using the following program: 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min.
3. Amplified DNA is verified by electrophoresis of aliquots of PCR mixture (2 μL) in 1.5% agarose in 1 $\times$ TAE buffer.
4. A 50 μL aliquot of the 16S rRNA gene solution is mixed with 30 μL of PEG solution and 12 μL of 3 M sodium acetate, gently shaken for 10 min at room temperature, and centrifuged at >16,000*g* for 15 min.
5. The supernatant is removed carefully by pipetting and then precipitated DNA is washed twice with 70% ethanol (*see Note 7*) and redissolved in 20 μL of sterile distilled water. Purified 16S rRNA genes are stored at –20°C until analysis.

3.2.2. T-RFLP Analysis for ABI PRISM 310 Genetic Analyser

The following protocol can be used in the ABI PRISM 310 Genetic Analyzer, ABI PRISM 3100 Genetic Analyzer and ABI 3130xl Genetic Analyzer instruments. Any modification specific for each instrument is also noted.

1. Purified PCR product (2 μL) is digested with 20 U of *Hha*I, *Msp*I, *Alu*I, *Hae*III, or *Rsa*I (Takara Bio or Toyobo, Japan) in a total volume of 10 μL at 37°C for 3 h.
2. The restriction digest product (1 μL) is mixed with 12 μL of Hi-Di Formamide and 1 μL of DNA fragment length standard. The standard size marker is a 1:1 mixture of GS 500 ROX and GS 1000 ROX. In the case of ABI 3130xl Genetic Analyzer, GS 1200 LIZ is used as a standard size marker.
3. Each sample is denatured at 95°C for 2 min and then immediately placed on ice.
4. The length of T-RF is determined on an ABI PRISM 310 Genetic Analyser (Applied Biosystems) in GeneScan mode (15 kV, 8 μA and 60°C for 48 min for each sample). 310 Capillary 47 cm and 310 POP-4 are used (*see Note 8*).
5. Fragment sizes are estimated by using the Local Southern Method in GeneScan 3.1 software (Applied Biosystems) (*see Note 9*).

6. T-RFs with a peak area of less than 25 fluorescence units are excluded from the analysis. In the case of ABI 3100 and 3130xl Genetic Analyzers, T-RFs with a peak area of <2% of the total area are excluded.
7. Fragments are resolved to one base pair by manual alignment of the size standard peaks from different electropherograms.
8. Predicted T-RFLP patterns of the 16S rRNA genes of known bacterial species are obtained using the GENETYX-MAC program (Software Developing Co., Tokyo) (*see Note 10*). An example is given in **Fig. 6.1**.

3.3. Denaturing Gradient Gel Electrophoresis

The electrophoresis gel is made of polyacrylamide (acrylamide and bis-acrylamide) containing a linear gradient of the denaturants urea and formamide, increasing from the top of the gel to the bottom. The experiments are carried out in a DcodeTM Universal Mutation Detection System (Bio-Rad, Richmond, VA). The gradient of denaturants can be adjusted according to the fragment of DNA amplified by PCR. The example we give here is of a polyacrylamide gel with a linear gradient of denaturant ranging from 30 to 70%, formed from mixing stock solutions of polyacrylamide (6%): (a) a solution containing 0% UF and another containing 100% of denaturants UF (100% corresponds to 7 M urea and 40% of deionized formamide).

3.3.1. PCR Amplification of 16S rRNA Gene

1. Amplification reactions are performed in a total volume of 50 μ L containing 5 μ L of DNA extract (100 ng), 0.2 μ M of each primer, 5 μ L of 10 $\times$ PCR buffer, 2.5 mM MgCl₂, 1.5 units *Taq* DNA polymerase, and 0.2 mM of each dNTP, and deionized water to make up the volume.
2. 16S rRNA genes are amplified in an Eppendorf Thermocycler (Hamburg, Germany) using the following program: initial denaturation step at 94°C for 3 min, 36 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final extension step of 72°C for 10 min.

Prior to DGGE analysis, the presence of PCR products is confirmed by electrophoresis in a 1.5% agarose gel conducted at 4 V/cm in Tris–borate–EDTA buffer. The gel is stained for 15 min with 0.5 μ g/mL ethidium bromide and viewed under ultraviolet transillumination. A 100-bp DNA ladder (New England Biolabs, Beverly, MA, USA) serves as the molecular size standard.

3.3.2. DGGE Analysis

1. The following instructions assume the use of a DcodeTM Universal Mutation Detection System. They can be easily adapted to other DGGE instruments.
2. Pouring the gel: Two glass slabs are used in the assembly of the gel. Assemble the slabs using the spacers and clamps as

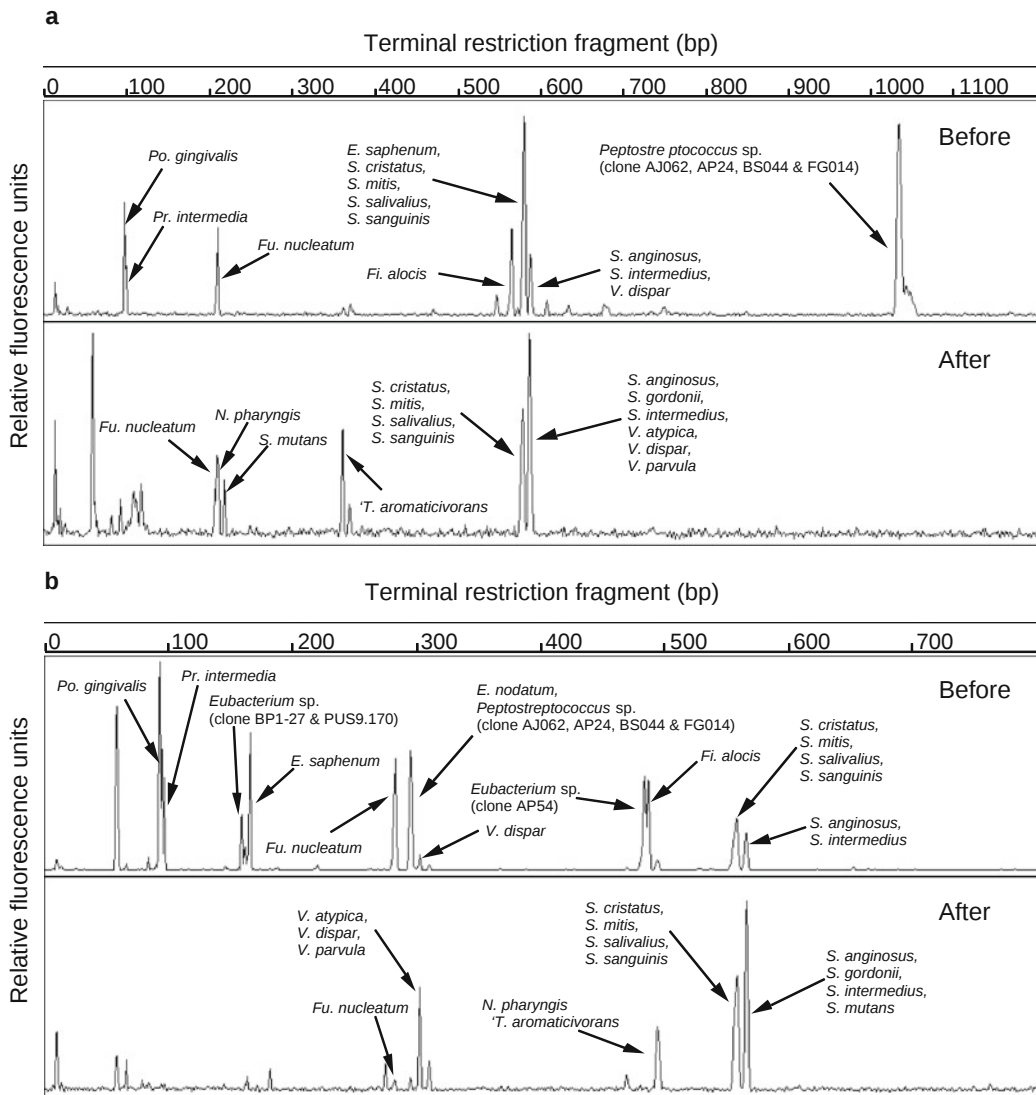


Fig. 6.1. Terminal restriction fragment length polymorphism patterns of 16S rRNA genes from subgingival plaque samples of a patient with periodontitis taken before treatment and after treatment generated after digestion with *HhaI* (A) and *MspI* (B). 16S rRNA genes were amplified with universal primers 27F and 1492R. Almost all the terminal restriction fragments were presumed to be species or phylotypes detected by the 16S rRNA gene clone library analysis. *E.*, *Eubacterium*; *Fi.*, *Filifactor*; *Fu.*, *Fusobacterium*; *N.*, *Neisseria*; *Po.*, *Porphyromonas*; *Pr.*, *Prevotella*; *S.*, *Streptococcus*; *T.*, “*Terrahaemophilus*”; *V.*, *Veillonella*. Reproduced with permission from Sakamoto et al. (3). © (2004) Society for General Microbiology.

depicted in **Fig. 6.2**. It is highly recommended to clean the trays with gauze and alcohol. Do not use cotton or paper, because their residue can fluoresce under exposure to UV light.

3. Insert the alignment card to keep the spacers parallel and push the slabs and spacers down and to each other at the same time you squeeze both clamps (not too tight).

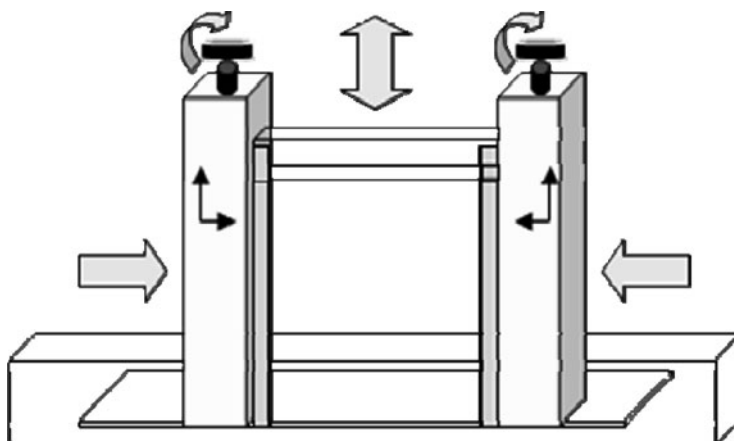


Fig. 6.2. General alignment of the DGGE plates.

4. Remove the card from the rack and check if the alignment was correct, moving a finger under the borders to see the alignment.
5. Place the sandwich on the gray sponge into the casting stand and turn the handles down to lock the assembly in place. Find the comb you want to use and place it near the stand.
6. Preparation of the denaturant gradient (prepare these solutions with the 0% UF and 100% UF solutions) (*see Note 11*): Prepare 12 mL of the lower and upper gradient solutions in separate Falcon tubes (marked L and H), being UF low (for example 30%), UF high (for example 70%). Also prepare a tube with 5 mL of 0% solution for the stacking gel.
7. Add 30 μL of gel-dye (without glycerol) in 12 mL of the solution with the highest concentration of denaturants (the gel coloration will present a gradient of blue color, corresponding to the gradient concentration).
8. After preparation of the denaturing solutions, add the APS and TEMED quickly to prevent polymerization of the gel in the syringe. Add 4.2 μL of 10% APS and 0.83 μL of TEMED per mL of solution. Thus, each 12 mL of solution will take 50 μL of 10% APS and 10 μL of TEMED. These reagents are responsible for polymerization of acrylamide and bis-acrylamide (polymerization begins immediately after addition of TEMED, so work quickly).
9. Draw each solution into the syringes. For our gels, the low denaturant syringe is on the front side of the gradient maker and the high denaturant is on the backside. Make sure air is removed from syringes (avoid bubble

formation). The needles are then coupled to the device that will form the gradient (gradient wheel from Bio-Rad) along the denaturant gel.

10. Set up the gradient former by inserting 30 mL syringes into a “closed” gradient wheel. Secure the syringes and “back out” the syringe plungers by reversing the gradient wheel. Note the resulting volume measured on each syringe (between 12 mL), which will be the amount of solution that the system will deliver to create the new gel. Immediately wash the syringes with distilled water after the use, to avoid polymerization of the remaining solutions.
11. Place the delivery tube in between the two plates near the center of the top edge of the plate assembly. Slowly and consistently turn the wheel until the gel is poured (**Fig. 6.3A**).
12. Finally, a solution without denaturing agents completes the gel (stacking gel) and where the comb is coupled (after

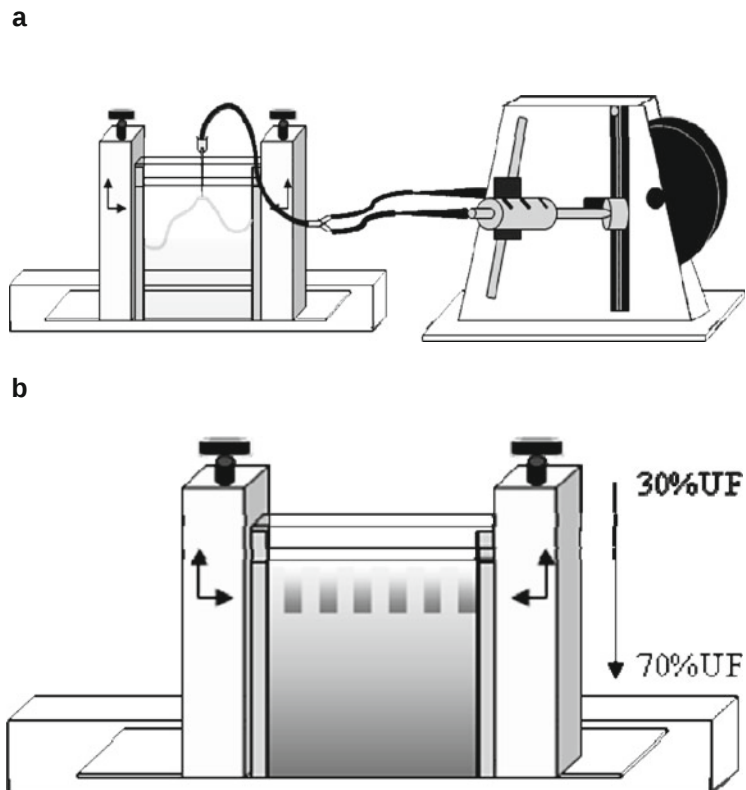


Fig. 6.3. Preparation of denaturant gradient. **A** The lower and the upper gradient solutions are loaded into separate syringes. **B** Denaturing gel with different UF concentrations – UF low (e.g., 30%) and UF high (e.g., 70%).

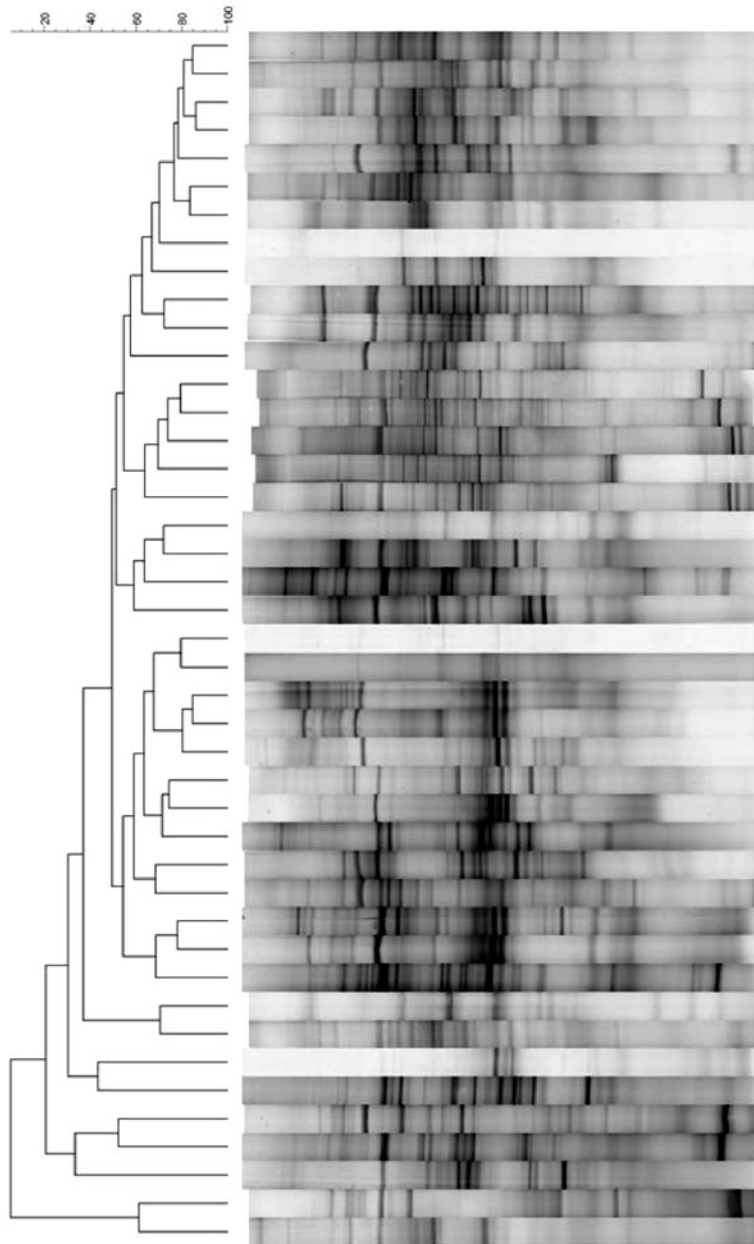


Fig. 6.4. Dendrograms obtained by the UPGMA method for clustering of DGGE-banding patterns of oral samples using the Gel Compar II (version 5.10.) software package.

polymerization, the comb is removed and the slots will be formed) (**Fig. 6.3B**).

13. Allow the gel to polymerize for about 2 h. Then, it must be mounted in the DGGE device, which permits two gels to run simultaneously (if you are running only one gel, use glass slabs without spacers to close the system and fit it

- in the support). After polymerization, remove the comb carefully. Rinse the slots to remove non-polymerized gel with $0.5 \times$ TAE buffer.
14. Running the gel: Add 7 L of fresh $1 \times$ TAE buffer to the buffer tank up to the “fill” mark. Switch on the DCode™ Universal Mutation Detection System (Bio-Rad) at least 60 min before electrophoresis so that the buffer can heat up to 60°C (it is not needed to activate the stirrer underneath the tank and the pump of the Dcode™ system).
 15. Load about 20 μL of PCR products (*see* **Note 12**) and 20 μL of loading buffer (1:1). The application of the samples should occur with the “slots” previously immersed in $1 \times$ TAE (running buffer).
 16. Load a marker sample along with the test samples (for determination of band positions and comparisons between different gels using software (e.g., Gel Compar II), we use three marker lanes: one in the middle slot and one at each slot at the extremities of the gel).
 17. Run the gel at 60°C for either 16 h at 75 V or 4 h at 200 V.
 18. After running, turn off the power supply, remove the control unit, and take out the core. Remove the sandwich and the clamps and remove one glass plate.
 19. Gels are stained with SYBR™ Green I nucleic acid gel stain (Molecular Probes) for 40 min and then scanned using a Storm PhosphorImager (Amersham Biosciences, Uppsala, Sweden).
 20. The digitized images of DGGE gels can be analyzed using the Gel Compar II software (Applied Maths, Kortrijk, Belgium) or other approaches (6) (**Fig. 6.4**).

4. Notes

1. We have also had good results using the QIAamp DNA Mini Kit, following the recommendations of the manufacturer (Qiagen, Valencia, CA).
2. 6-FAM-labeled forward primer 8f is light sensitive. It must be stored in the dark at -20°C .
3. PEG solution is very viscous.
4. The condition of the capillary will significantly affect the T-RFLP patterns.
5. During preparation and development of DGGE gels, highly toxic and carcinogenic materials are used. Always

wear a lab coat, gloves, and a mask during all steps and change them even when the slightest contamination is suspected. Use blue nitrile gloves when handling acrylamide/bis-acrylamide solutions.

6. Store the bottles in the dark at 4°C. For good quality gels do not use a 100% denaturant solution which is older than 1 month.
7. The precipitated DNA may be easily washed out from microcentrifuge tube. It is important for a good result to ensure recovering of the precipitated DNA.
8. The length of T-RF is influenced by the type of genetic analyzer, capillary, polymer, and size standard. In the case of ABI 3100 and 3130xl Genetic Analyzers, the length of T-RF is determined in GeneScan mode (15 kV, 100 μ A, and 60°C for 40 min for each sample) and GeneMapper mode (8.5 kV, 100 μ A and 60°C for 112 min for each sample), respectively. 3130xl & 3100 Capillary Array 36 cm and 3100 POP-4 (for 3100 Genetic Analyzer) and 3130xl & 3100 Capillary Array 50 cm and 3100 POP-7 (for 3130xl Genetic Analyzer) are used.
9. In the case of ABI 3100 and 3130xl Genetic Analyzers, fragment sizes are estimated by using the Local Southern Method in GeneScan 3.7 and GeneMapper 4.0 softwares (Applied Biosystems), respectively.
10. There are size discrepancies between predicted and observed T-RF lengths (1).
11. Example of calculations to prepare a gel with gradient denaturant (gradient 30–70% UF): prepare the 30% UF solution of denaturant (final volume of 12 mL) using the formula:

$$C_i \times V_i = C_f \times V_f, \quad [1]$$

where C_i is the initial concentration of 100% denaturant solution, V_i is the volume necessary to reach the 30% UF concentration, C_f is the final concentration of the solution (in this example, 30% UF denaturant), and V_f is the final volume of the solution (here we are using 12 mL for one syringe). We have:

$$100 \times V_i = 30 \times 12. \quad [2]$$

In the above example, 3.6 mL from the 100% UF solution will be added to 8.4 mL of 0% denaturant solution (to complete the total volume of 12 mL of denaturant solution).

Prepare 70% UF solution of denaturant (final volume of 12 mL). Here, just replace the value of C_f in the equation (1) by 70. Thus, we have:

$$100 \times V_i = 70 \times 12. \quad [3]$$

The value of V_i , therefore, will be 8.4 mL. To complete the volume of 12 mL, add 3.6 mL of 0% denaturant solution. Note that these calculations are valid for the construction of a gel with gradient denaturant from 30 to 70%. These values may be adjusted in the formula (1) to make gels with a range of values of denaturation.

12. The quantity of PCR products applied to the gel may vary depending on the quality of amplicons and the sensitivity of the method used for gel staining.

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References

1. Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I., and Benno, Y. (2003) Application of terminal RFLP analysis to characterize oral bacterial flora in saliva of healthy subjects and patients with periodontitis. *J. Med. Microbiol.* **52**, 79–89.
2. Zijnge, V., Harmsen, H. J., Kleinfelder, J. W., van der Rest, M. E., Degener, J. E., and Welling, G. W. (2003) Denaturing gradient gel electrophoresis analysis to study bacterial community structure in pockets of periodontitis patients. *Oral Microbiol. Immunol.* **18**, 59–65.
3. Sakamoto, M., Huang, Y., Ohnishi, M., Umeda, M., Ishikawa, I., and Benno, Y. (2004) Changes in oral microbial profiles after periodontal treatment as determined by molecular analysis of 16S rRNA genes. *J. Med. Microbiol.* **53**, 563–571.
4. Siqueira, J. F., Jr, Rôças, I. N., and Rosado, A. S. (2004) Investigation of bacterial communities associated with asymptomatic and symptomatic endodontic infections by denaturing gradient gel electrophoresis fingerprinting approach. *Oral Microbiol. Immunol.* **19**, 363–370.
5. Rôças, I. N., Siqueira, J. F., Jr, Aboim, M. C., and Rosado, A. S. (2004) Denaturing gradient gel electrophoresis analysis of bacterial communities associated with failed endodontic treatment. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **98**, 741–749.

6. Siqueira, J. F., Jr, Rôças, I. N., and Rosado, A. S. (2005) Application of denaturing gradient gel electrophoresis (DGGE) to the analysis of endodontic infections. *J. Endod.* **31**, 775–782.
7. Li, Y., Ku, C. Y., Xu, J., Saxena, D., and Caulfield, P. W. (2005) Survey of oral microbial diversity using PCR-based denaturing gradient gel electrophoresis. *J. Dent. Res.* **84**, 559–564.
8. Sakamoto, M., Rôças, I. N., Siqueira, J. F., Jr, and Benno, Y. (2006) Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. *Oral Microbiol. Immunol.* **21**, 112–122.
9. Zijlge, V., Welling, G. W., Degener, J. E., van Winkelhoff, A. J., Abbas, F., and Harmssen, H. J. (2006) Denaturing gradient gel electrophoresis as a diagnostic tool in periodontal microbiology. *J. Clin. Microbiol.* **44**, 3628–3633.
10. Machado de Oliveira, J. C., Siqueira, J. F., Jr, Rôças, I. N., Baumgartner, J. C., Xia, T., Peixoto, R. S., and Rosado, A. S. (2007) Bacterial community profiles of endodontic abscesses from Brazilian and USA subjects as compared by denaturing gradient gel electrophoresis analysis. *Oral Microbiol. Immunol.* **22**, 14–18.
11. Siqueira, J. F., Jr, Rocas, I. N., Debelian, G. J., Carmo, F. L., Paiva, S. S., Alves, F. R., and Rosado, A. S. (2008) Profiling of root canal bacterial communities associated with chronic apical periodontitis from Brazilian and Norwegian subjects. *J. Endod.* **34**, 1457–1461.
12. Alves, F. R., Siqueira, J. F., Jr, Carmo, F. L., Santos, A. L., Peixoto, R. S., Rocas, I. N., and Rosado, A. S. (2009) Bacterial community profiling of cryogenically ground samples from the apical and coronal root segments of teeth with apical periodontitis. *J. Endod.* **35**, 486–492.
13. Liu, W. T., Marsh, T. L., Cheng, H., and Forney, L. J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63**, 4516–4522.
14. Clement, B. G., Kehl, L. E., De Bord, K. L., and Kitts, C. L. (1998) Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J. Microbiol. Methods.* **31**, 135–142.
15. Spiegelman, D., Whissell, G., and Greer, C. W. (2005) A survey of the methods for the characterization of microbial consortia and communities. *Can. J. Microbiol.* **51**, 355–386.
16. Matsumoto, M., Sakamoto, M., Hayashi, H., and Benno, Y. (2005) Novel phylogenetic assignment database for terminal-restriction fragment length polymorphism analysis of human colonic microbiota. *J. Microbiol. Methods.* **61**, 305–319.
17. Sakamoto, M., Umeda, M., and Benno, Y. (2005) Molecular analysis of human oral microbiota. *J. Periodontal. Res.* **40**, 277–285.
18. Gasser, R. B. (1998) What's in that band? *Int. J. Parasitol.* **28**, 989–996.
19. Myers, R. M., Fischer, S. G., Lerman, L. S., and Maniatis, T. (1985) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res.* **13**, 3131–3145.
20. Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.
21. Muyzer, G., and Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek.* **73**, 127–141.
22. Machado de Oliveira, J. C., Gama, T. G., Siqueira, J. F., Jr, Rocas, I. N., Peixoto, R. S., and Rosado, A. S. (2007) On the use of denaturing gradient gel electrophoresis approach for bacterial identification in endodontic infections. *Clin. Oral Invest.* **11**, 127–132.

Chapter 7

Protocols to Study the Physiology of Oral Biofilms

José A. Lemos, Jacqueline Abranches, Hyun Koo,
Robert E. Marquis, and Robert A. Burne

Abstract

The oral cavity harbors several hundred different bacterial species that colonize both hard (teeth) and soft tissues, forming complex populations known as microbial biofilms. It is widely accepted that the phenotypic characteristics of bacteria grown in biofilms are substantially different from those grown in suspensions. Because biofilms are the natural habitat for the great majority of oral bacteria, including those contributing to oral diseases, a better understanding of the physiology of adherent populations is clearly needed to control oral microbes in health and disease. In this chapter, we use oral streptococci as examples for studying the physiology of oral biofilms.

Key words: Biofilm, oral streptococci, *Streptococcus*, enzymatic assays, stress, production of polysaccharides.

1. Introduction

Oral biofilms normally exist in dynamic equilibrium with host defenses and are important for preventing colonization by undesirable organisms (1, 2). However, changes in the composition and metabolic activities of biofilm communities that lead to increases in the proportion of pathogenic species can lead to oral diseases, including dental caries and periodontitis. Despite the importance of oral biofilms to health and disease, studies on the physiology and genetics of oral bacteria were primarily conducted using planktonic populations of bacteria. In recent years, the development of in vitro and in vivo biofilm methodologies to study sessile populations have demonstrated that there are many

physiologic and molecular differences between planktonic and surface-bound bacteria, suggesting that the organisms can acquire a “biofilm phenotype” (2–4).

Starting with the premise that “all models are wrong, but some are useful,” a quote attributed to the British statistician George Box, there are a variety of in vitro systems to study oral streptococci biofilms. These include simple and economical models in which bacteria are cultivated in batch systems using different surfaces such as glass, plastic, or hydroxyapatite (HA), the latter being used as a surrogate of tooth enamel. These systems can give reproducible results and can be scaled up to provide sufficient biomass for physiologic and genetic studies. In addition to batch and static systems, the use of shear force in continuous flow systems is considered ideal for the analysis of the dynamics of cell attachment to surfaces and the initial stages of biofilm development. Yet another commonly used system is the so-called constant depth film fermentor in which a scraper intermittently passes over the grown biofilms in wells to achieve constant biofilm depth. Batch and continuous feed systems can also be used to generate more complex multispecies biofilms of known composition, or microcosms can be formed from starter biofilm samples from the body and subsequent cultivation of biofilms in vitro. Because of the natural heterogeneity of these more complex biofilms, the interpretation of the behavior of these populations is very challenging.

Here, we focus on batch-culture systems that our laboratories have routinely used for studying the physiology of oral biofilms, with a particular emphasis on *Streptococcus mutans* biofilms. One of the advantages of the models presented in this chapter is that multiple biofilms can be formed simultaneously, which provides significant benefit in establishing reproducibility of the data and reducing variance. In addition, test agents can be applied and removed from the system instantaneously allowing a tightly controlled substance exposure time. Moreover, biofilms formed on glass slides or HA are amenable to confocal and electron microscopy and can yield a quantity of bacterial biomass that is sufficient for enzymatic assays. Finally, these model systems can be easily adapted for studies with non-streptococcal species.

2. Materials

2.1. Biofilm Medium (BM)

Base medium (5) per liter

K ₂ HPO ₄	10 g
KH ₂ PO ₄	2 g

$(\text{NH}_4)_2\text{SO}_4$	1.3 g
NaCl	2 g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.02 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.001 g
Casamino acids	2 g

Dissolve all components in deionized (Milli-Q) water. Autoclave (121°C for 20 min) and store at room temperature (*see Note 1, Note 2*).

100× amino acid stock solution	per 100 mL
L-glutamate (L-glutamic acid)	5 g
L-arginine·HCl	2 g
L-cysteine·HCl	2 g
L-tryptophan	0.2 g

Dissolve all components in Milli-Q water. Filter-sterilize ($0.22\ \mu\text{m}$ pore size) and store wrapped in aluminum foil (components are light-sensitive) at 4°C for up to 4 weeks.

100× vitamin stock solution	per 100 mL
Pyridoxine HCl	240 mg
Nicotinic acid	46 mg
Pantothenic acid	24 mg
Riboflavin	4 mg
Thiamine HCl	1 mg
D-Biotin	0.12 mg

Dissolve all components with Milli-Q water. Filter-sterilize ($0.22\ \mu\text{m}$ pore size) and store wrapped in aluminum foil at 4°C .

Final BM medium composition	per liter
Base medium	950 mL
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g/mL stock)	5 mL
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.03 g/mL stock)	5 mL
100× vitamin stock	10 mL
100× amino acid stock	10 mL
1 M glucose or 0.5 M sucrose (<i>see Note 3</i>)	20 mL

Adjust mixed solution to pH 7 and filter-sterilize ($0.22\ \mu\text{m}$ filter). Use immediately or store wrapped in aluminum foil at 4°C for up to 1 week.

2.2. Tryptone-Yeast Extract (TY) Medium

per liter

Bacto tryptone	30 g
Bacto yeast extract	5 g

Dissolve in Milli-Q water, autoclave (121°C , 20 min), and adjust pH to 7 aseptically with NaOH. Add glucose or sucrose (20% stock solution) to a final concentration of 1% after autoclaving to avoid caramelization.

2.3. Low Molecular Weight (LMW) Medium

per liter

Bacto tryptone	25 g
Bacto yeast extract	15 g

Filter solution through a Millipore Prep/Scale-TFF cartridge (10 kDa cut-off) using a peristaltic pump. Add KH_2PO_4 (25 mM final concentration) and MgSO_4 (4 mM final concentration) and adjust the pH to 7. Autoclave (121°C, 20 min). Add glucose or sucrose (20% stock solution) to a final concentration of 1%.

2.4. Adsorption Buffer (AB)

50 mM KCl, 1 mM potassium phosphate (0.35 mM K_2HPO_4 plus 0.65 mM KH_2PO_4), 1 mM CaCl_2 , 0.1 mM MgCl_2 . Adjust pH to 6.5. Store at room temperature.

2.5. Clarified Saliva

Collect 50 mL of whole saliva on ice from one donor. Mix saliva with AB buffer (1:1 ratio, v/v). Add 50 μL 0.1 M phenylmethylsulfonyl fluoride (PMSF, store at 4°C for up to 9 months). Centrifuge the mixture (5,500g, 4°C, 10 min). Collect supernatant (clarified whole saliva) and filter through a 0.22 μm PES low protein-binding filter.

2.6. Buffers

1. Glycine buffer. 0.1 M glycine adjusted to desired pH with 1 N HCl or 1 N KOH.
2. Phosphate buffer (pH 7): Mix 60 mL of 1 M K_2HPO_4 with 40 mL of 1 M KH_2PO_4 . The pH of the mixed solution should be pH 7.

2.7. 2× ATPase Assay Buffer

100 mM Tris-maleate buffer (pH 7). Prepare a 200 mM stock solution of Trizma-maleate. Adjust 50 mL of stock solution to desired pH with 0.1 M NaOH. Make up to 100 mL with Milli-Q water.

2.8. Bencini Reagent

100 mM ZnCl_2 plus 15 mM ammonium molybdate.

2.9. Salt Solution

50 mM KCl plus 1 mM MgCl_2 .

2.10. Other Materials or Equipment

1. 96-well, flat-bottom microtiter plate (Costar, Corning Inc. USA) – required when saliva-coated plates are used for biofilm growth (*see* below).
2. One milliliter (1 mL) disposable cuvettes for spectrophotometric readings.
3. Camera for photographing biofilms.
4. Hydroxyapatite disks with a surface area of $2.7 \pm 0.2 \text{ cm}^2$ (Clarkson Chromatography Products, Inc., South Williamsport, PA, USA) – this is required for growing biofilms on a hydroxyapatite matrix.

5. 24-well tissue culture plate (Corning, NY, USA).
6. Ultrasonic bath.
7. 14-mL (Falcon) centrifuge tubes.
8. 50 mL conical tubes.
9. Sonicator (e.g., Branson Sonifier 150 or equivalent).
10. pH probe to measure pH changes in broth media.
11. Dissolved oxygen meter (e.g., VWR Model 4000).
12. Acid-washed 0.1 mm diameter glass beads (Biospec, Bartlesville, OK).
13. Beadbeater (e.g., Biospec, Bartlesville, OK) for homogenizing cells.
14. Vacuum concentrator (e.g., SpeedVac).
15. A desiccator containing phosphorus pentoxide (P_2O_5) or Drierite[®]. (under vacuum) for drying samples.

2.11. Other Chemicals or Media

1. 0.1% crystal violet: Dissolve 0.1 g crystal violet in 100 mL deionized water.
2. 33% acetic acid: To 33 mL glacial acetic acid, add deionized water to make up to 100 mL.
3. 0.89% NaCl (filter-sterilized or autoclaved)
4. Brain heart infusion (BHI) medium (per liter): 37.5 g broth powder. Autoclave for 20 min at 121°C to sterilize. If solid medium is required, add agar to 15 g/L.
5. Toluene–acetone (1:9 ratio).
6. 20% tricarboxylic acid (TCA).
7. 100 mM adenosine triphosphate (ATP), pH 7.0.
8. 2 mM NADH for NADH assays.
9. Bovine liver catalase (50 mg/mL stock) for NADH assays.
10. 0.2% iodine in 2% potassium iodide solution – required for measuring intracellular polysaccharides.

3. Methods

3.1. Biofilm Growth

3.1.1. Quantitative Growth of Biofilms on Microtiter Plates

This method is particularly useful to assess the ability of different strains to form biofilms (*see Note 4*).

1. Prepare overnight cultures, in triplicates, in 5 mL of TY plus 1% glucose [or Brain Heart Infusion (BHI)] broth and incubate at 37°C in a 5% CO₂ atmosphere.

2. Transfer 100 μL of the overnight culture to a tube containing 5 mL of pre-warmed fresh TY (or BHI) medium and incubate at 37°C in a 5% CO_2 atmosphere to an optical density at 600 nm (OD_{600}) of 0.5. Chill on ice and keep cells on ice until the last culture reached the desired OD_{600} .
3. Prepare fresh BM containing the desired amount of carbohydrate and pre-warm media at 37°C for 1 h.
4. *Optional.* Coat the wells of a 96-well, flat-bottom microtiter plate (Costar 3595, Corning Inc. USA) with 50 μL of clarified saliva for 1 h at 37°C . Remove unbound saliva by blotting the plate on a clean absorbent paper.
5. Dilute cultures 1:100 in fresh BM containing the desired carbohydrate source. Dispense 200 μL of each diluted culture into six wells. Wells containing uninoculated growth medium should serve as negative controls.
6. Incubate plate for 24 h at 37°C in a 5% CO_2 atmosphere without agitation.
7. *Optional.* To measure total growth yield of each strain, remove both planktonic and biofilm cells by scraping the bottom of *one* well using a pipette tip. Transfer to a 1 mL cuvette. Blank with one of the cuvettes containing uninoculated medium.
8. Blot the plate on a paper towel to remove culture media. To remove loosely bound cells, carefully immerse the microtiter plate in a large dish with distilled water. Blot the plate on a paper towel. Repeat this step twice.
9. Add 50 μL of 0.1% crystal violet to the test wells, including the negative control wells. Incubate for 15 min.
10. Repeat step 8.
11. Air dry plate and photograph.
12. Add 200 μL of 33% acetic acid solution to the wells. Leave for 10 min. Keep the plate covered. Transfer the acetic acid solution to a 1 mL cuvette (*see Note 5*).
13. Bring the volume of all cuvettes to 1 mL using Milli-Q water (including controls). Mix well.
14. For biofilm growth, read at a wavelength of 575 nm (OD_{575}). Blank with a cuvette containing the solution from a crystal violet-stained uninoculated well.

3.1.2. Growth of Biofilms in Gram-Staining Boxes

This method is excellent for experiments that normally require a large bacterial biomass, particularly enzymatic assays.

1. Prepare overnight cultures in 10 mL of TY broth and incubate overnight at 37°C in a 5% CO_2 atmosphere.



Fig. 7.1. Typical *Streptococcus mutans* mature biofilm grown on glass slides placed in a Gram-staining box using TY medium supplemented with 1% sucrose.

2. Autoclave a Gram-staining box (*see Fig. 7.1*) containing a slide holder basket and the desired number of microscope glass slides.
3. Fill the sterile Gram-staining box to completely cover the slides with TY broth containing 1% (w/v) of the desired (tested) carbohydrate.
4. Add 10 mL of the overnight culture and incubate for 48 h at 37°C in a 5% CO₂ atmosphere.
5. Aseptically transfer the slide holder to another sterile Gram-staining box filled with medium (*see Fig. 7.1*). From now on, repeat this step once a day for 4–5 days (*see Note 6*).

3.1.3. Growth and Processing of Biofilms on Saliva-Coated Hydroxyapatite (HA) Disks

This method uses hydroxyapatite disks coated with saliva (mimicking the presence of salivary pellicle), placed in a vertical position.

1. Select HA disks and place it in the custom-made holder (*see Fig. 7.2*).

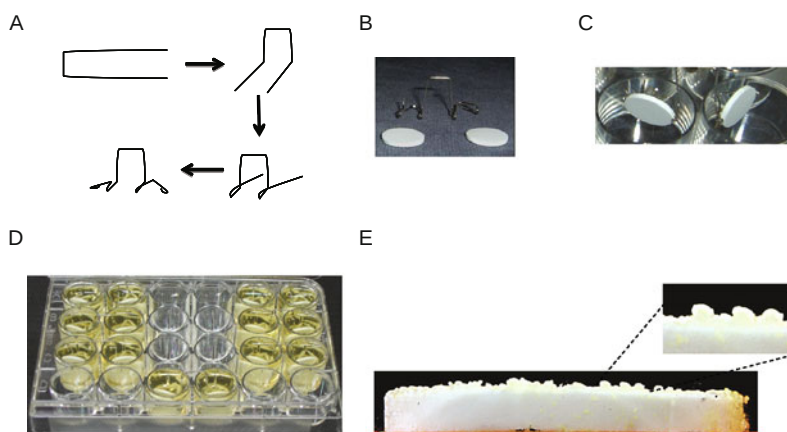


Fig. 7.2. Growth of biofilm on saliva-coated hydroxyapatite (SHA) disks. **A** Schematic showing how to make the disk holder using orthodontic wire, **B** hydroxyapatite disks and custom-made holder, **C** SHA disks placed in a vertical position inside the wells of a microtiter plate, **D** growth of biofilms using LMWS medium, **e** typical 5-day-old biofilm formed on SHA disk.

2. Place the disks vertically (attached in the holder) into a 24-well culture plate. Pipette clarified saliva into wells until disks are submerged (approximately 2.8 mL saliva per well).
3. Incubate disks in saliva at 37°C for 1 h on an orbital shaker. Remove disks, shake off excess saliva, and dip-wash once in sterile AB buffer. The saliva-coated HA (sHA) disks are now ready for use.
4. Prepare overnight culture in 5 mL of LMW medium containing 1% glucose (LMWG). Dilute overnight culture 1:20 in LMWG and grow the cells at 37°C in 5% CO₂ to an OD₆₀₀ of 0.5.
5. Dilute culture 1:250 in LMW containing 1% sucrose (LMWS: 3 mL of medium per disk), which will provide approximately 5×10^6 colony-forming units (CFU) per mL. Transfer 2.8 mL of the inoculated medium into a 24-well tissue culture plate. Place a freshly prepared sHA disk into each well. Check the disk position (should be vertical) and the disks should be completely submerged. Incubate the plate for 24 h at 37°C in 5% CO₂.
6. After 24 h incubation, transfer each of the sHA disks to a new plate containing fresh LMWS. Repeat this procedure daily for 4–6 days. Biofilms are considered mature after day 4.
7. Biofilm processing: Dip-wash the mature biofilms in 0.89% NaCl solution three times. Carefully release each disk from the holder and drop it into a sterile glass tube containing 1 mL of sterile 0.89% NaCl. Pipette an additional 1 mL of 0.89% NaCl to each tube. Place the tubes in an ultrasonic bath for 10 min.
8. Remove each disk from its tube using a spatula and check if all biomass has been removed.
9. Transfer suspension of glass tubes to 14-mL sterile centrifuge tubes. Wash each glass tube with 1 mL of sterile 0.89% NaCl using a pipettor and transfer the content to the centrifuge tube. Repeat this procedure two more times. Final volume of cell suspension should be approximately 5 mL.
10. Place tubes on ice. Immerse the tip of the probe of a sonicator at least 1 in. deep into the solution (without touching the bottom or the side walls). Sonicate the samples with three 30-s pulses at an output of 7 W.
11. The biofilm homogenate can be used for several assays, including determination of biomass (dry weight), protein content, and extracellular and intracellular polysaccharide composition (*see* **Section 3.7** below).

3.2. Acid-Mediated Killing

Since most microbes in nature grow in biofilms, the use of biofilms for assessing cidal action is generally more appropriate than the use of suspensions. The example presented here is for acid-mediated killing. This assay can be performed with intact biofilms grown on the surface of microtiter plates for 24–48 h, glass slides, or HA disks. Below we describe a standard protocol using biofilms grown on microtiter plates.

1. Grow biofilms for 24 h using BM medium containing the desired sugar source as described in **Section 3.1.1** (*see Note 7*).
2. Blot the plate on absorbent paper, e.g., paper towel, to remove culture media.
3. Trying not to disturb the biofilm, slowly add 200 μL of 0.1 M glycine buffer adjusted to pH 2.7 to each well (*see Note 8*). The control biofilm group should receive 200 μL of 0.1 M glycine buffer pH 7 and processed in the first and last time points.
4. At each desired time point, remove biofilm by scraping the bottom with a pipette tip and transfer cell suspension into a microfuge tube and disperse cells either by vortexing at maximum speed or by sonication for 30 s.
5. Prepare serial dilutions in sterile 1% Difco peptone broth of dispersed cells and plate dilutions in triplicate in Brain Heart Infusion plates.
6. Incubate plates for 24–48 h at 37°C in a 5% CO_2 atmosphere before colonies are counted. Cell viability at each time point is expressed as the log percentage of viable cells [colony-forming units (CFU) per mL] relative to the control group at time zero. The killing course should follow the relationship $N = N_0 e^{kt}$, where N_0 is the initial count (in CFU/mL) at time zero, N is the count at time t , t is the sampling time, e is the base for natural logarithms, and k is the killing rate constant. An alternative equation is $N = N_0 2.3 \log_{10} kt$.

3.3. pH Drop (Glycolytic Profile)

1. Grow biofilms on glass slides in Gram-staining boxes for 5–6 days as described in **Section 3.1.2**.
2. Remove slides from the box with a sterile forceps and dip three times into a 50 mL sterile conical tube containing 45 mL of 50 mM KCl/1 mM MgCl_2 solution.
3. Transfer the slide to a 50 mL conical tube containing 49 mL of 50 mM KCl/1 mM MgCl_2 solution and a small magnetic stir bar (the volume of salt solution can be reduced to prevent overflow). Place the tube in a beaker on a stir plate.

4. Insert a pH probe into the KCl/MgCl₂ solution and titrate the solution pH to 7.2 using 0.1 N KOH (it may take several min to stabilize the pH at 7.2). Reduce agitation if biofilms start to detach from the glass slides.
5. Add 900 μ L of 1 M glucose. The final concentration of glucose in the tube should be 55.6 mM. Record the pH drop every 30 s for the first 5 min and then, every 5 min for the next 85 min.

3.4. F-ATPase Assay

1. Scrape biofilm cells from the glass slides or HA disks into a 50 mL conical tube containing 5 mL of 1 $\times$ ATPase buffer and sonicate the cell suspension as described in step 10 of **Section 3.1.3**.
2. Centrifuge at 4,000*g* for 12 min at 4°C. Carefully discard the supernatant. Resuspend pellet in 5 mL of 1 $\times$ ATPase buffer and repeat centrifugation.
3. Resuspend the pelleted cells in 2 mL of 1 $\times$ ATPase buffer.
4. Chill the cells on ice and add 100 μ L of toluene–acetone (1:9).
5. Vortex for 2 min, then chill on ice for 2 min. Repeat.
6. Freeze the cells in dry ice ethanol bath and thaw at 37°C. Repeat.
7. Into a glass tube, pipette 0.75 mL of 2 $\times$ ATPase buffer, 0.475 mL of Milli-Q water, 0.2 mL of permeabilized cells. Mix well and incubate in the water bath at 37°C for 2 min.
8. Then, add 75 μ L of pre-warmed 100 mM ATP (pH 7) solution.
9. Incubate at 37°C for 15 min.
10. Stop the reaction by adding 0.5 mL 20% tricarboxylic acid (TCA). Centrifuge for 14,000*g* for 10 min. Collect the supernatant.
11. For the blank tube (negative control), do the same procedure, but add TCA at time zero to inactivate the enzyme first.
12. To quantify the inorganic phosphate released by the ATPase, prepare the following mix in a 2 mL plastic cuvette (light path = 1 cm):
 - 0.15 mL H₂O
 - 0.15 mL supernatant containing ATP
 - 0.9 mL of Bencini reagent

Mix well and read the absorbance at 350 nm (A_{350}). Calculate the ΔA_{350} ($\Delta A_{350} = A_{350}$ of experimental – A_{350} of control).

13. Phosphate concentration can be calculated with use of a standard curve of inorganic phosphate prepared in the same way as described above using the concentration range from 0.5 to 6 μg of standard phosphate in the reaction mixture.
14. To calculate the results, One unit of ATPase activity is defined as the amount of the enzyme that can release 1 μmol phosphate per min. Relative rates can be expressed per unit of dry biomass or per mg of protein.

3.5. Respiration

1. Grow biofilms on glass slides in Gram-staining boxes for 5–6 days as described in **Section 3.1.2** (*see Note 9*).
2. Remove slides from the box with a sterile forceps and wash biofilms rapidly by immersion in salt solution (50 mM KCl/1 mM MgCl_2).
3. Transfer the slide to a 50 mL conical tube containing approximately 35 mL of air-saturated 50 mM potassium phosphate buffer pH 7 containing 0.5% (wt/vol) glucose.
4. Start measuring the oxygen uptake at room temperature by using a dissolved oxygen meter (e.g., VWR Model 4000).
5. Record the readings every min for 15 min or until the reading approaches zero.
6. After readings are completed, collect 10 μL of cell suspension to assess protein concentration by using standard protein concentration assays.
7. Calculate the respiration rate by estimating the value differences between each interval (rate = $\text{nmol O}_2/\text{min}/\text{mg}$ of protein).

3.6. NADH Oxidase Activity

1. Grow biofilms on glass slides for 5–6 days as described in **Section 3.1.2**.
2. Remove slides from the medium with a sterile forceps and scrape biofilms with a sterile spatula into a 50-mL conical tube containing 10 mL of 20 mM Tris-HCl buffer (pH 7) containing salt solution.
3. Sonicate as described in **Section 3.1.3**.
4. Centrifuge at 4,000*g* for 12 min at 4°C and carefully discard supernatant.
5. Resuspend cell pellet in 10 mL of salt solution and repeat steps 3 and 4 once.
6. Resuspend pellet in 600 μL salt solution.
7. Transfer pellet to a 1.5 mL screw cap tube containing approximately 600 μL of acid-washed 0.1 mm diameter glass beads.

8. Homogenize the cells by using a Beadbeater for six cycles of 30 s with incubation on ice for 2 min between cycles.
9. Centrifuge at 14,000*g* for 10 min at 4°C.
10. Collect the clear supernatant. This is your crude extract and it should be kept on ice to prevent proteolysis.
11. In a 2 mL plastic cuvette (1 cm light path), prepare the following mix: 100 µL of 1 M potassium phosphate buffer (pH 7), 6 µL of 50 mM EDTA, 10 µL of 50 mg/mL bovine liver catalase, and 10–100 µL of crude extract. Bring the volume to 920 µL Milli-Q water.
12. Blank the spectrophotometer at 340 nm with the cuvette containing the reagents listed above. Start the reaction by adding 80 µL of 2 mM NADH.
13. Record the decrease in absorbance at 340 nm (OD_{340}) every 15 s for 3 min, during which time the reaction is linear.
13. Use 10 µL of the crude extract to obtain protein concentration using standard methods.
14. One unit of NADH oxidase is defined as the amount of enzyme that catalyzed the oxidation of 1 µmol NADH/min. NADH activity is measured as (units/mg protein).

3.7. Determination of Extracellular Polysaccharides (EPS)

1. Use a biofilm homogenate obtained as described in **Section 3.1.3** (total volume of 5 mL). Remove 100 µL for total bacterial counting.
2. Centrifuge at 5,500*g* for 10 min at 4°C and carefully pour the supernatant into a 50 mL conical tube (save this tube to pour additional supernatant as follow).
3. Wash the pellet by pipetting 2.6 mL of Milli-Q water and vortex each tube until the pellet is completely dissolved.
4. Centrifuge at 5,500*g* for 10 min at 4°C. Pour supernatant in the conical tube from step 2. Repeat the wash with 2.5 mL water, as described above once more. Total volume of supernatant should be 10 mL. *Save supernatant for soluble polysaccharide analysis (see below).*
5. Resuspend the pellet in 2.55 mL water. Remove 50-µL aliquot (for determination of total protein) and dilute 1:5 in water. Pipette 50 µL of the diluted sample in micro-glass vial (in triplicates). Spin samples in a vacuum concentrator (SpeedVac) for approximately 1 h. Place tubes with the dried samples in a desiccator containing P₂O₅ (phosphorus pentoxide) or Drierite[®] under vacuum. Total protein in each

sample is determined by acid digestion followed by the ninhydrin assay (6).

3.7.1. Determination of Soluble EPS

1. To determine soluble EPS, transfer 3 mL from the supernatant obtained in step 4 to a 15-mL conical tube. Add 2.5 volumes of ice-cold 95% ethanol (7.5 mL), mix well, and place in freezer (-20°C) overnight to precipitate the water-soluble polysaccharides. The amount of supernatant should be enough to perform in triplicates.
2. Remove the samples from freezer, mix well by inverting at least five times, and centrifuge all the tubes at $9,500g$ for 20 min at 4°C . Discard the supernatant in a clean glass beaker (make sure the supernatant is clear).
3. Wash pellets three times with ice-cold 75% ethanol and resuspend pellet in 7 mL ice-cold 75% ethanol. Use a disposable inoculating loop to scrape down the water-soluble EPS adhered to the tube wall.
4. Vortex mixture well and centrifuge ($9,500g$ for 20 min at 4°C). Discard supernatant. Repeat this procedure two more times.
5. After washing, blot-dry the pellet.
6. Resuspend pellet in 1 mL water by scraping down the sides. Vortex to bring pellet back into solution.
7. Determine total glucose and fructose using colorimetric assays (7, 8).

3.7.2. Determination of Insoluble EPS

1. Take 1-mL aliquot of the resuspended pellet from step 5 of **Section 3.7** and centrifuge immediately ($16,000g$ for 10 min at 4°C). Carefully remove supernatant of the tubes with a tip connected to a vacuum pump. Spin the samples in a SpeedVac vacuum concentrator for 2 h. Place tubes with the dried pellets in a desiccator containing P_2O_5 or Drierite[®] under vacuum until ready for analysis.
2. Weigh each sample pellet and transfer to a 1.5-mL microfuge tube. According to the weight recorded, add $300\ \mu\text{L}$ of 1 N NaOH per mg dry weight to each tube. Vortex gently to disrupt cell pellet. Incubate at 37°C for 2 h.
3. During the first extraction, after 30 min, remove tubes from the incubator and vortex each tube until the pellet is completely dissolved. Place tube back in the incubator and complete the 2 h incubation time.
4. After 2 h, centrifuge the samples at $14,000g$ for 10 min. Transfer supernatant to a 1.5-mL tube. *Do not* discard pellet.

5. Again, add same volume of 1 N NaOH and vortex to disrupt pellet. Repeat all steps as described above.
6. Resuspend the pellet in NaOH, vortex, and centrifuge. Combine supernatant from each extraction in the same tube.
7. Neutralize pH of the extract by slowly adding 1 N HCl stepwise and checking the pH constantly until you reach $\text{pH } 7 \pm 0.5$. After neutralizing the sample, add 3 volumes of ice-cold 95% ethanol, mix well, and precipitate the EPS in the freezer overnight (at least 18 h incubation).
8. After precipitation, remove tubes from freezer, mix well, and centrifuge samples for 20 min (9,500g, 4°C). Discard supernatant.
9. Resuspend pellet in 7 mL of ice-cold 75% ethanol. Use a disposable inoculating loop to scrape down the polysaccharide adhered to the tube wall to ensure resuspension. Vortex, centrifuge for 20 min (9,500g, 4°C), and discard supernatant. Repeat this procedure two more times.
10. After washing, blot-dry the pellet.
11. Resuspend pellet by scraping down the sides in 1 N NaOH (in the same total volume of the original extraction).
12. Determine total glucose and fructose using colorimetric assays as described elsewhere (7, 8).

3.7.3. Determination of Intracellular Polysaccharides (IPS)

1. Take 1-mL aliquot of a biofilm previously dispersed by sonication as described above (*see* step 5 of **Section 3.7**) and immediately centrifuge at 13,000g for 10 min at 4°C.
2. Carefully remove supernatant of the tubes with a tip connected to a vacuum pump. Spin samples in a SpeedVac vacuum concentrator for 2 h.
3. Place tubes with the dried pellets in a desiccator containing P₂O₅ or Drierite[®] under vacuum.
4. Weigh each pellet as described before and transfer to a clean glass culture tube (18 × 150 mm, previously rinsed with Milli-Q water).
5. According to the weight recorded for each pellet, add 1 mL water per 1 mg dry weight to each tube. According to the volume added, add 300 µL 5.3 M KOH per mL cell suspension. Make sure pellets are completely disrupted.
6. Tightly cover the tubes, individually, with aluminum foil and boil in water for 1.5 h.

7. After boiling, let tubes cool down. Add 5.3 M HCl to each tube in the same volume as the KOH to neutralize suspension.
8. The IPS concentration will be determined by a colorimetric assay (9) as follows:
 In a 2-mL tube prepare the following mix:
 - (a) 800 μ L sample or standard (0–100 μ g glycogen) (*see Note 10*).
 - (b) 500 μ L 1 M phosphate buffer pH 7. Vortex to mix.
 - (c) 300 μ L 0.2% iodine in 2% potassium iodide solution.
 Vortex each tube well and read absorbance at 520 nm (A_{520}).

4. Notes

1. Unless stated otherwise, all media and chemical reagents are from Difco or Sigma, respectively.
2. Some precipitation in base medium can be observed because of the presence of FeSO_4 and MnCl_2 .
3. Lower or higher concentrations of sugars can be used so that growth can be catabolite limited or pH limited.
4. This protocol can be adapted for physiologic assays that require a larger biomass using tissue culture plates.
5. For sucrose grown *S. mutans* biofilms, an additional acetic acid extraction might be required.
6. Biofilms are considered mature after 5- to 6-days growth. Depending on the protocol requirements, disrupted or intact biofilms can be used in the physiologic assays.
7. To minimize experimental variations, three independent wells containing intact biofilms should be used for each time point.
8. Changes as low as 0.1 pH unit result in great variations in the killing kinetics of different species of oral streptococci. A pilot experiment to determine the best pH and sampling times is recommended.
9. Dispersed biofilms can also be used.
10. The glycogen solution should always be prepared fresh.

Acknowledgments

We thank Dr. Pedro Rosalen for kindly providing images used in Fig. 7.1.

References

1. Wade, W. (1999). Unculturable bacteria in oral biofilms, in *Dental plaque revisited. Oral biofilms in health and disease* (Newman, H. N., and Wilson, M., Eds.). Boline, Cardiff, pp. 313–322.
2. Stoodley, P., Sauer, K., Davies, D. G., and Costerton, J. W. (2002) Biofilms as complex differentiated communities. *Ann. Rev. Microbiol.* **56**, 187–209.
3. Lemos, J. A., Abranches, J., and Burne, R. A. (2005) Responses of cariogenic streptococci to environmental stresses. *Curr. Issues Mol. Biol.* **7**, 95–107.
4. Stewart, P. S., and Franklin, M. J. (2008) Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* **6**, 199–210.
5. Loo, C. Y., Corliss, D. A., and Ganeshkumar, N. (2000) *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. *J. Bacteriol.* **182**, 1374–1382.
6. Moore, S., and Stein, W. H. (1954) A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. Biol. Chem.* **211**, 907–913.
7. Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A., and Smith, F. (1951) A colorimetric method for the determination of sugars. *Nature.* **168**, 167.
8. Kulka, R. G. (1956) Colorimetric estimation of ketopentoses and ketohexoses. *Biochem. J.* **63**, 542–548.
9. DiPersio, J. R., Mattingly, S. J., Higgins, M. L., and Shockman, G. D. (1974) Measurement of intracellular iodophilic polysaccharide in two cariogenic strains of *Streptococcus mutans* by cytochemical and chemical methods. *Infect. Immun.* **10**, 597–604.

Chapter 8

Adhesion of Yeast and Bacteria to Oral Surfaces

Richard D. Cannon, Karl M. Lyons, Kenneth Chong,
and Ann R. Holmes

Abstract

Colonization of surfaces in the human body by microorganisms is an early, essential, step in the initiation of infectious disease. We have developed in vitro assays to investigate interactions between yeast or bacterial cells and human tissues, fluids, or prostheses. Such assays can be used to identify the adhesins, ligands, and receptors involved in these interactions, for example by determining which components of the microbe or human tissue/fluid interfere with adherence in the assay. The assays can also be applied to finding ways of preventing adhesion, and subsequent disease, by investigating the effects of different conditions and added compounds on adherence in the in vitro assays. We describe six assays for measuring adhesion of the oral yeast *Candida albicans*, a common commensal and opportunistic pathogen, or the bacterium *Staphylococcus epidermidis*, which is not normally pathogenic but is known to form biofilms on medical prostheses. The assays described represent two approaches to investigating adhesion; retention at a fixed time point following liquid washes; and retention against a continuous flow of medium.

Key words: *Candida albicans*, *Staphylococcus epidermidis*, adhesion, saliva, colonization, epithelial cells, silicone, hydroxyapatite, polymethyl methacrylate.

1. Introduction

The oral cavity provides many surfaces and niches which are colonized by a variety of microorganisms that form complex biofilms (1). While these microbial communities and biofilms are often non-pathogenic, certain microorganisms play important roles in oral diseases such as dental caries, periodontitis, and oral candidiasis. It is therefore important to investigate the multiple microbial/host adhesion interactions within the oral cavity, such as those that promote development of the oral biofilm dental plaque,

and those that facilitate colonization by pathogenic microorganisms implicated in oral diseases (2). In some instances it may be possible to preclude diseases by inhibiting microbial adhesion and preventing colonization. Many bacteria, fungi, and viruses adhere to host tissues and other substrates by lectin-like interactions and carbohydrates are under development as drugs to interfere with the microbial–host interaction (3, 4). *Candida albicans* is a commensal yeast normally present in the oral cavity in low numbers relative to bacterial species. It is also an opportunistic pathogen that causes oral candidiasis and interfering with its adhesion has the potential to prevent such infections (5). Probiotics, which are used to reduce or prevent colonization by pathogenic microbes, can act by inhibiting adhesion (6).

In order to develop novel antimicrobial strategies that prevent or reduce adhesion, microbial adhesion must be assessed and monitored using appropriate assays. In this chapter we describe the assays used in our laboratory to measure the adhesion of the oral yeast *C. albicans* and the skin commensal bacterium *Staphylococcus epidermidis* to different model surfaces that represent those found in the oral environment. Our assays use radiolabeling of the yeast/bacteria, or microscopy, to detect and quantify the interactions with various substrates. Although we focus on two species, the assays described are applicable to studies of the adhesion of other oral microorganisms.

There are two main categories of adhesion assays: one measures microbial retention at a fixed time point following liquid washes; the other measures adhesion and retention against continuous liquid flow in real time. We describe examples of both. An important aspect of both assays is the nature of the force challenging the retention of the microbe on, or with, the substrate: in the fixed time point static assay, this is the wash condition before adhesion is measured; for the flow assay, it is the shear force applied as adhesion is measured. Selection of the type of assay to be used depends on the *in vivo* system being modeled. In the oral cavity, where initial adherence is often a matter of retention on a saliva-coated surface, both types of assay have relevance. Use of a parallel plate flow chamber with continuous liquid flow (7) has the advantage that adhesion can be studied quantitatively with *in situ*, real-time, observation, without use of radioactive compounds. It is also possible to control the hydrodynamic conditions in terms of shear rate, flow velocity, and Reynolds number, which determine the mechanism of mass transport. In addition, the passage of air/liquid interfaces across the substratum for rinsing, fixation, and/or staining purposes can be avoided in continuous flow systems. The non-flow systems have the advantages of not requiring complex equipment and allowing multiple assays in small volumes, yet they remain relevant to *in vivo* conditions.

In general, radiolabeling is our first choice for detecting or quantifying adhesion. This approach provides high sensitivity and reproducibility compared to quantifying attachment by either microscopy or viable cell count. In many cases assays can be designed for a microtiter plate format to enable simplification of the protocol and to reduce reagent use.

We describe six representative assays of microbial adhesion developed in our laboratory. The assays measure adhesion of *C. albicans* in static assays to the following substrates: (i) saliva-coated hydroxyapatite (HA) (8), (ii) immobilized saliva proteins (*C. albicans* overlay assay) (9, 10), (iii) epithelial cells following saliva treatment of the yeast cells (11), (iv) saliva-coated medical grade silicone (12) or saliva-coated denture prosthetic material (Holmes, Lyons et al., unpublished data). Other assays measure adhesion of *S. epidermidis* to denture prosthetic material under (i) static or (ii) flow conditions (Lyons et al., unpublished data).

2. Materials

2.1. Radiolabeling of Yeast and Bacterial Cells and Cell Culture

1. Equipment (additional to usual laboratory equipment): scintillation counter with a microplate and microfuge tube capability (*see* **Note 1**). Anaerobic jar for the cultivation of bacteria and GasPak EZ anaerobic container system (Becton Dickinson & Co [BD], Franklin Lakes, NJ, USA).
2. Radiochemicals (*see* **Notes 2** and **3**): (a) ^{35}S -methionine: Yeast cells are labeled with ^{35}S -methionine (EasyTagTM L- ^{35}S]-Methionine, stabilized aqueous solution; PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA). (b) ^3H -thymidine: bacterial cells are labeled with ^3H -thymidine ([methyl- ^3H] thymidine, aqueous solution, sterilized; GE Healthcare UK Ltd, Chalfont St Giles, UK). Both are stored refrigerated. Concentrations of radiochemicals required for each adherence assay vary and are detailed in **Section 3**.
3. Yeast strains. For most assays, several yeast species and strains, including laboratory strains as well as clinical isolates, have been used successfully. The most commonly used strain in our laboratory is *C. albicans* ATCC 10261 (American Type Culture Collection, Manassas, VA). The materials and methods described are applicable for other yeast species.
4. Media and buffers. All media and buffers are made with water purified by reverse osmosis with a resistivity of 10–15 M Ω cm.
5. Yeast culture media: (a) Yeast extract peptone dextrose (YEPD) liquid medium is used to prepare the strains for

storage and contains per liter: 10 g yeast extract (BD), 20 g bacto peptone (BD), and 20 g glucose (final concentration 111 mM). For agar plates, 20 g agar (Danisco NZ Ltd, East Tamaki, NZ) is added; (b) Glucose salts biotin (GSB) liquid medium is used to prepare inocula and contains per liter: 1 g $(\text{NH}_4)_2\text{SO}_4$ (7.57 mM), 2 g KH_2PO_4 (14.7 mM), 50 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 mM), 50 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.34 mM), 0.05 mg biotin, and 20 g glucose (111 mM) (*see* **Note 4**).

6. Bacterial strains. For the assays described, we used *S. epidermidis* strains ATCC 12228, ATCC 14990, and ATCC 49134 and clinical isolates from the oro-nasal cavity of an individual following a maxillary resection. However, we have successfully used strains of other bacterial species, such as *Staphylococcus aureus* and various oral streptococcal species, in similar adhesion assays and the method of radio-labeling the bacterial cells is the same.
7. Bacterial culture medium: (a) Brain heart infusion–yeast extract (BHY) liquid medium contains per liter: 37 g brain heart infusion (BD) and 5 g yeast extract (BD); (b) Tryptone yeast extract (TY) medium: dissolve the following in 987.5 mL water, 5 g tryptone (BD), 5 g yeast extract, 4 g K_2HPO_4 , autoclave, then add 12.5 mL filter sterilized 40% (w/v) glucose (final concentrations in medium: K_2HPO_4 , 23 mM; glucose, 27.8 mM); (c) TY-B consists of sterile TY and BHY mixed aseptically 20:1 (v/v).

2.2. Blot Overlay

Assay to

Demonstrate

Adhesion of Yeast

Cells to Immobilized

Proteins

1. Equipment (additional to usual laboratory equipment) that may be required: polyacrylamide gel electrophoresis (PAGE) apparatus and electro-transfer (Western blotting) apparatus (*see* **Note 5**); end-over-end mixer; access to dark room facilities and photographic equipment and chemicals.
2. Yeast strains and media: *C. albicans* ATCC 10261 is grown in GSB and labeled with ^{35}S -methionine as described in **Section 3.1**.
3. PAGE separation of proteins: materials required include (i) Separating buffer: 18.2 g Tris (final concentration 1.5 M), 0.4 g SDS (final concentration 14 mM) per 100 mL water, adjust to pH 8.8 with HCl. Filter solution through Whatman No. 1 paper and store at 4°C; (ii) Stacking buffer: 6.1 g Tris (final concentration 0.5 M), 0.4 g SDS (final concentration 14 mM) in 100 mL water; adjust pH to 6.8 with HCl; filter solution through Whatman No. 1 paper and store at 4°C; (iii) Acrylamide–bis solution (40%, 37.5:1; Bio-Rad, Hercules, CA) (hazard – *see* **Note 6**) (iv) Tetramethylethylenediamine (TEMED; Bio-Rad); (v) 10% ammonium persulfate (*see* **Note 7**); (vi) Loading dye (contains in 10 mL: 50 mg bromophenol blue, 3 mL water,

10 μ L 1 M NaOH, and 7 mL glycerol); (vii) Sample buffer (10 mL stock contains 2.5 mL stacking gel buffer, 0.2 mL 2-mercaptoethanol, 1 mL 20% (w/v) SDS, and 6.3 mL water) store as frozen aliquots; (viii) Bradford protein assay kit (Bio-Rad); (ix) Pre-stained protein markers (*see Note 8*) of size range appropriate for proteins to be separated (Invitrogen, Carlsbad, CA, USA); (x) Electrophoresis running buffer (contains per liter: 14.4 g glycine (192 mM), 3.03 g Tris (25 mM); 1 g SDS (3.5 mM)); (xi) Coomassie Blue protein stain contains per liter: 0.2 g Coomassie Blue R250, 400 mL methanol, 500 mL water, and 100 mL glacial acetic acid; (xii) Destain solution contains per liter: 200 mL methanol, 700 mL water, 100 mL glacial acetic acid.

4. Electroblothing of PAGE-separated proteins and blot overlay: materials required include (i) Nitrocellulose membrane (Hybond ECL, GE Healthcare UK Ltd); (ii) 3MM chromatography paper (Whatman); (iii) Transfer buffer, contains per liter: 3.03 g Tris (25 mM), 14.4 g glycine (192 mM), 200 mL methanol (20% v/v) (*see Note 9*); (iv) KCl buffer (1 L) made up of solution A containing in 800 mL water: 0.27 g KH_2PO_4 (final concentration in complete buffer 2 mM), 0.46 g K_2HPO_4 (final concentration in complete buffer 2.6 mM), 0.373 g KCl (final concentration in complete buffer 5 mM), pH 6.5, and solution B containing 0.15 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (final concentration in complete buffer 1 mM) in 200 mL water; solutions A and B are autoclaved separately before mixing to prevent precipitation; (v) Blocking buffer: 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, USA) in KCl buffer; (vi) Blot-staining solution: 0.2% Ponceau S in 1% (v/v) acetic acid; (vii) Phosphate-buffered saline (PBS) contains per liter: 8.5 g NaCl (145.5 mM), 0.3 g KH_2PO_4 (2.2 mM), 0.6 g Na_2HPO_4 (4.2 mM), pH 7.5; (viii) Tween 20 (Bio-Rad).
5. Detection of radioactivity by autoradiography: Dried membranes are incubated with X-ray film (e.g., Amersham Hyperfilm β max or similar film sensitive to ^{35}S radiation).

2.3. Adhesion of *C. albicans* Cells to Saliva-Coated Hydroxyapatite

1. Equipment (additional to usual laboratory equipment) that may be required: scintillation counter with microfuge tube capability.
2. Yeast strains and media: *C. albicans* ATCC 10261 is grown in GSB and labeled with ^{35}S -methionine as described in **Section 3.1**.
3. Buffers: KCl buffer (*see* Step 4 (iv) of **Section 2.2** above).
4. Saliva: whole saliva (approximately 10 mL, unstimulated) is collected on ice from five donors and an equal amount from

each donor is pooled. The pooled saliva sample is clarified by centrifugation at 12,000*g* for 15 min and the supernatant is mixed with an equal volume of KCl buffer. Proteinase inhibitors are added (use a commercially available cocktail such as SigmaFAST; Sigma Aldrich). Care – infectious disease hazard, take standard precautions for handling and disposal of human biological material.

5. Hydroxyapatite beads: Portions (12 mg) of hydroxyapatite beads (Macro-Prep Ceramic Hydroxyapatite Type I, 80 μ m, Bio-Rad) are hydrated prior to use in the adhesion assay by static incubation in 1.5 mL microfuge tubes in 0.5 mL KCl buffer at 4°C for 16 h.
6. Scintillation fluid (for example, Optiphase 3, PerkinElmer).

2.4. Adhesion of Saliva-Treated *C. albicans* Cells to Epithelial Cells

1. Equipment (additional to usual laboratory equipment) that may be required: biohazard hood with sterile air flow; inverted microscope; hemocytometer; CO₂ incubator; confocal microscope (*see Note 10*); 12-channel multichannel pipette; scintillation counter with a microplate capability.
2. Yeast strains and media: *C. albicans* ATCC 10261 is grown in GSB and labeled with ³⁵S-methionine as described in **Section 3.1**.
3. Epithelial cells and culture media: human epithelial cell lines HEp-2 and A549 were obtained from the European Collection of Cell Cultures, Centre for Applied Microbiological Research, Salisbury, UK. Cells are grown in Eagles MEM medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen) and 1% glutamine (recently thawed commercially available solution: 200 mM, Sigma Aldrich, stored at –20°C as 100× stock). Confluent cells are prepared for subculture with trypsin–EDTA (commercially available solution containing 0.05% Trypsin 0.53 mM EDTA, Invitrogen, stored at –20°C).
4. Saliva samples are prepared as described above (Step 4 of **Section 2.3**).
5. Sterile tissue culture flasks for tissue culture maintenance: Nunclon™ cell culture flasks, area 80 cm² (Nunc, Thermo Fisher Scientific, Roskilde, Denmark).
6. Sterile 96-well flat bottom microtiter culture plates with lids for adherence assays with radiolabeled cells (Nunc).
7. Sterile 24-well flat bottom culture plates with lids (Nunc) for confocal microscopy with fluorescein isothiocyanate (FITC)-labeled cells.
8. Buffers: (i) Artificial saliva buffer (ASB) constituted to mimic the ionic composition of saliva contained per liter: 1.36 g KH₂PO₄ (10 mM); 0.29 g CaCl₂·2H₂O (2 mM); 1.36 g

KCl (18.2 mM); 0.028 g NaSCN (0.35 mM); 0.63 mg NaF (15 μ M); 2.18 g NaHCO₃ (26 mM; pH 6.5); and glucose (1 g (5.6 mM)); (ii) PBS (*see* Step 4 (vii) of **Section 2.2**); (iii) carbonate/bicarbonate buffer pH 9.5 (contains per liter: 3.18 g Na₂CO₃ (30 mM), 5.88 g NaHCO₃ (70 mM) [*see* **Note 11**]).

9. Scintillation fluid.

**2.5. Adhesion
of *C. albicans*
or *S. epidermidis*
Cells to Saliva-
Coated Medical
Grade Silicone
or to Denture
Prosthetic Materials**

1. Equipment (additional to usual laboratory equipment) that may be required: scintillation counter with a microfuge tube capability; microtome.
2. Yeast strains and media: *C. albicans* ATCC 10261 is grown in GSB and labeled with ³⁵S-methionine as described in **Section 3.1**.
3. Bacterial strains and medium: *S. epidermidis* is grown in TY-B and radiolabeled with ³H-thymidine as described in **Section 3.1**.
4. Silicone: Medical grade silicone is cut from standard, single consistency silicone blocks (Silimed Silastic, InterMed Medical Ltd, Auckland, NZ) into 3 cm × 3 cm × 1 cm blocks which are fixed onto plastic tissue embedding cassettes (Tissue-Tek Uni-Cassette, Sakura Finetek USA, Inc., Torrance, CA, USA) using Araldite[®] Ultra Clear epoxy adhesive (Selley's Pty. Ltd, Padstow, NSW, Australia). The fixed silicone block is sliced (1 cm × 3 cm) to a thickness of 300 μ m using a microtome. The silicone slices are then cut by hand with sharp blade to make three 1 cm × 1 cm × 300 μ m coupons from each slice (*see* **Note 12**).
5. Denture prosthetic materials: commonly used denture prosthetic materials, which are also used to make interim and final obturator denture prostheses, have been tested in our laboratory using this assay. They include heat-polymerized polymethyl methacrylate (Vertex Regular and Vertex Implacryl, Vertex Dental, Zeist, The Netherlands); chair-side cold-cure polymethyl methacrylate (Kooliner, GC America, Inc., Illinois, USA; Vertex Castapress, Vertex Dental; and New Rimseal, Bosworth Company, Illinois, USA); tissue conditioners (Viscogel, Dentsply; Softliner, GC America, Inc.); silicone-based chair-side lining material (Silagum, DMG, Hamburg, Germany); and laboratory polymerized silicone (Molloplast-B, Bolton Dental Manufacturing, Ontario, Canada). Circular coupons of these materials are made using a gypsum mold with 20 patterns measuring 5 mm diameter × 2 mm thick. Where appropriate, the materials are polymerized in the molds according to the manufacturer's recommendations. After removal of the coupons from the gypsum mold, excess material is removed using a

laboratory handpiece (KaVo EWL K9, KaVo Dental GmbH, Biberach, Germany) and a tapered carbide bur (GEBR Bräsele GmbH & Co KG, Lemgo, Germany) prior to adhesion testing.

6. Saliva and “saliva wash”: Saliva is prepared as described in Step 4 of **Section 2.3**; saliva wash (*see* **Note 13**) is prepared by rinsing the mouth for 30 s with food grade water (20 mL) before collection into a 50 mL Falcon tube.
7. Scintillation fluid.

2.6. Adhesion of *S. epidermidis* Cells to Denture Prosthetic Materials Under Flow Conditions

1. Equipment (additional to usual laboratory equipment) that may be required: parallel plate flow chamber and inverted phase-contrast microscope (*see* **Notes 14** and **15**).
2. Bacterial strain: *see* Step 6 of **Section 2.1**.
3. Flow adhesion buffer (FAB) contains per liter: 3.73 g KCl (50 mM), 0.17 g K₂HPO₄ (1.0 mM), 0.14 g KH₂PO₄ (1.0 mM), 0.14 g CaCl₂ (anhydrous) (1.26 mM); pH 6.8.
4. Gypsum molds.
5. Standard glass microscope slides (75 mm × 25 mm × 1 mm and 75 mm × 25 mm × 0.8 mm).
6. Vaseline.
7. Dental yellow stone.
8. Dual-channel variable speed pump (Monostat Vera varistatic pump, Cole-Parmer, Vernon Hills, IL, USA).
9. Tubing, 0.89 mm diameter (platinum-cured silicone tubing, Cole-Parmer).
10. Digital SLR camera (Nikon D70s digital SLR camera, Nikon Corporation, Tokyo, Japan) with microscope mountings.
11. Image analysis software, Fovea Pro 4 (Reindeer Graphics, Inc., Asheville, NC, USA) which operates in the Photoshop CS2 environment on Apple computers.

3. Methods

3.1. Radiolabeling of Yeast and Bacterial Cells and Cell Culture

Yeast and bacteria for use in adhesion experiments are conveniently stored as glycerol stocks at −80°C. These cells can then be used to inoculate medium for growth of cells and for subsequent radiolabeling.

3.1.1. To Prepare Inocula for Pre-culture of Yeast or Bacteria

1. Yeast cells are streaked on YPD agar plates and incubated at 30°C for 24 h. Bacterial cells are streaked on BHY agar plates and incubated in an anaerobic jar at 37°C for 24 h.

2. Cells are removed from the surfaces of the YPD or BHY agar plates and resuspended in YPD or BHY containing 15% (w/v) glycerol (approximately 1 mL which will provide enough inoculum for at least 20 experiments), respectively, and stored at -80°C in 50 μL volumes in microfuge tubes.
3. Yeast inocula are tested by inoculating 50 mL GSB or YPD in 250 mL sterile conical flasks with 5–10 μL inoculum (diluted in sterile medium if required) and measuring the growth of cells (optical density at 540 nm [OD_{540}] using a pre-calibrated spectrophotometer (*see Note 16*)) during incubation at 30°C with shaking (200 rpm).
4. The inoculum volume used can be adjusted to get the required level of growth in a certain volume of medium after a particular incubation period.
5. Bacterial inocula are tested by inoculating 1.5 mL BHY in a microfuge tube with 1–5 μL inoculum (diluted in sterile medium if required) and measuring the growth of cells (optical density at 600 nm [OD_{600}]) during static incubation at 37°C .
6. The inoculum volume used can be adjusted to get the required level of growth in a certain volume of medium after a particular incubation period.

*3.1.2. Preparation
of C. albicans
Cells Radioactively
Labeled with
 ^{35}S -Methionine*

1. Cell cultures (50 mL, in 250 mL flask) are inoculated and grown in GSB at 30°C with shaking for approximately 16 h to a cell concentration of approximately 2.0×10^6 cells per mL, as determined by measurement of OD_{540} using a spectrophotometer and reference to a standard curve (*see Note 17*).
2. ^{35}S -methionine (2 μL , 20 μCi ; 1,175 Ci/mmol; *see Note 18*) is added to the flask which is incubated at 30°C with shaking for a further 2 h to allow incorporation of the radiolabel into the cells.
3. The cells are harvested by centrifugation (1,500*g*, 5 min) washed twice in 10 mL of adhesion assay buffer (e.g., KCl buffer) by centrifugation and following a determination of cell concentration by measuring the OD_{540} of a suitable dilution of cells, the cells are resuspended to the final cell concentration specified for the particular assay.

*3.1.3. Preparation of
S. epidermidis Cells
Radioactively Labeled
with ^3H -Thymidine*

1. Cell cultures (3 mL; two full microfuge tubes) are grown at 37°C for 16 h in TY medium containing [methyl- ^3H]-thymidine (10 μL , 10 μCi , 85 Ci/mmol, added to each tube).

2. Cells are harvested by centrifugation (4,000*g*, 5 min) and washed twice in KCl buffer by centrifugation before resuspending at the final cell concentration specified for the particular assay (determined by OD₆₀₀ measurement, as described above for yeast cells).

3.2. Blot Overlay Assay to Investigate Adhesion of Yeast Cells to Immobilized Proteins

In this assay, the protein to which adherence is to be determined (for example, a protein present in human saliva, *see* **Note 19**) is first subjected to PAGE separation, before electroblotting onto a nitrocellulose membrane which is then incubated with radio-labeled yeast cells. The protein bands to which the radiolabeled yeast have adhered are detected by autoradiography.

3.2.1. SDS-PAGE Analysis

1. Set up two SDS-PAGE gels using separating gels with an acrylamide concentration appropriate for the size of the protein to be detected (for example, for binding of *C. albicans* yeast cells to salivary proline-rich proteins, 10% gels are prepared).
2. The saliva or saliva rinse samples are diluted 50% with SDS-PAGE sample buffer and heated (80°C, 10 min) before loading onto the replicate gels (usually 15 µg total protein per lane, *see* **Note 20**). On each gel, load protein standards (5 µL) so that the molecular weight of separated proteins can be estimated.
3. The gels are placed in the apparatus (in the Bio-Rad apparatus the two gels are run back to back), submerged in running buffer, and subjected to direct current at a fixed voltage of 100 mV (10 mV/cm) (care – dangerous voltage, always disconnect power before disassembling apparatus) for approximately 90 min (until the blue dye front has reached the bottom of the gel).
4. Both gels are removed from the apparatus; one gel is stained to show the positions of the individual protein bands and the replicate gel is used for electroblotting.
5. Gel staining: one of several techniques can be applied depending on the expected concentration of the particular proteins in the sample. We describe here Coomassie Blue staining which is usually sufficiently sensitive. The gel is submerged in the stain solution and incubated at room temperature in a closed box with gentle agitation for at least 1 h (and up to 16 h if evaporation is prevented) and then developed by incubation at room temperature in destain solution with gentle agitation for 30–60 min or longer if required (*see* **Note 21**).
6. Gel-banding patterns are recorded by photography on a light box and protein bands can be quantified using commercially available computer programs or freely available

software such as NIH Image J (<http://rsb.info.nih.gov/ij/index.html>).

3.2.2. Electroblotting

1. Blotting is best done with a freshly run gel.
2. Pre-wet the cassette sponge pads, two pieces of 3MM chromatography paper, and the nitrocellulose membrane (cut to the same size as the gel) with transfer buffer.
3. Carefully use one piece of pre-wetted 3MM paper to remove the gel from the glass plate of the PAGE apparatus and place on top of one pre-wetted sponge pad and assemble the “sandwich” within the cassette in the following order: sponge pad, 3MM paper, gel, nitrocellulose, 3MM paper, and second sponge pad, and place in the electroblot apparatus and cover with transfer buffer.
4. The apparatus usually has an ice-pack in the tank and is run below 10°C (for example, in a cold room or refrigerated cabinet).
5. Voltage applied is usually 100 mV (10 mV/cm) for 90 min, but conditions can be varied for different sized proteins (longer for larger proteins which do not transfer easily; *see Note 22*).
6. Dismantle blotting apparatus and peel blot away from gel (the pre-stained markers allow visual confirmation of transfer) and store the blot between blotting paper or similar at 4°C until needed.

3.2.3. Radiolabeled Yeast Overlay

1. Block non-specific protein-binding sites on the nitrocellulose membrane by incubating it with BSA (5% w/v) in KCl buffer for 2 h at room temperature with reciprocal shaking (50–60/min). Dimensions of a suitable container (plastic or glass) should be sufficient in cross section to fit the blot with a space of approximately 1 cm around it and with a depth of 5–7 cm.
2. Remove the blocking solution, wash the blot with 50 mL KCl buffer, and submerge the blot in KCl buffer (30 mL) containing radiolabeled yeast cells at 1.1×10^7 cells/mL.
3. Incubate at 4°C with reciprocal shaking (50–60/min) for 16 h.
4. Remove yeast suspension (Care – radioactive material – dispose of according to appropriate regulations) and wash membrane four times (1 min) with PBS containing 0.1% (v/v) Tween 20 (40 mL) with gentle side-to-side manual agitation.
5. Remove blot with tweezers and air-dry on blotting paper prior to exposure to X-ray film. An example of an overlay blot is given in **Fig. 8.1**.

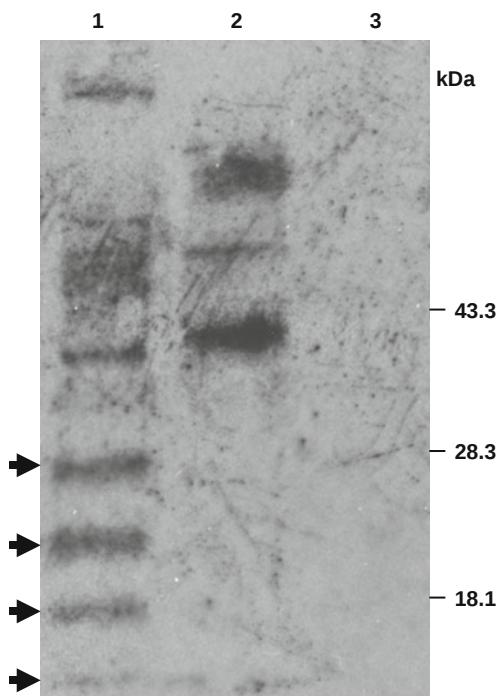


Fig. 8.1. Representative blot overlay assay autoradiogram showing adhesion of ^{35}S -methionine-radiolabeled *C. albicans* ATCC 10261 cells to PAGE-separated saliva samples. Samples were separated on a 10% polyacrylamide gel and electroblotted onto nitrocellulose before incubation with radiolabeled yeast cells and autoradiography. *Lane 1*, whole saliva; *lane 2*, sample extracted from saliva-coated dental acrylic; *lane 3*, control sample from untreated dental acrylic. Arrows (*lane 1*) indicate four components of whole saliva to which *C. albicans* adheres that have been identified as salivary basic proline-rich proteins (PRPs) (9). It can be seen that these proteins are not present in the sample of salivary pellicle from dental acrylic (*lane 2*).

3.3. Adhesion of *C. albicans* Cells to Saliva-Coated Hydroxyapatite

In this assay, hydroxyapatite beads are used as a model of the tooth surface, which is always coated in saliva (even after tooth cleaning procedures the tooth surface is rapidly coated with a salivary pellicle (13)).

1. Beads, prepared as described in **Section 2.3**, are incubated with 1 mL KCl buffer containing 0.1% (w/v) BSA with end-over-end mixing at 22°C for 1 h in order to block sites which bind proteins non-specifically.
2. The beads are then washed once with KCl buffer (1 mL) and then 0.9 mL KCl buffer and 0.1 mL radiolabeled cells (3.0×10^7 cells/mL in KCl buffer) are added to each tube.
3. The tubes are incubated at 22°C with end-over-end mixing for 90 min.
4. The liquid containing unattached cells is aspirated (Care – radioactive material – dispose of according to appropriate

regulations) and the beads are washed three times with KCl buffer (1 mL).

5. Scintillation fluid (1 mL) is added to the tubes and bead-associated radioactivity measured (*see* **Note 23**).

3.4. Adhesion of Saliva-Treated *C. albicans* Cells to Epithelial Cells

In this assay, monolayers of cultured epithelial cells are used in a model of *C. albicans* adherence to human mucosal surfaces. We have used cell lines from culture collections rather than primary cell cultures (for example, of oral epithelial cells) but the methods described could be applied to primary cell monolayers. In order to mimic intra-oral conditions, yeast cells are pre-treated with saliva before measuring adherence to the epithelial monolayers. Standard 96-well microtiter well culture plates are used for adherence assays using radiolabeled cells, and 24-well culture plates containing sterile glass coverslips are used for confocal microscopy analysis of adherence.

3.4.1. Epithelial Cell Monolayers

1. Cultures are passaged using standard aseptic techniques in a biological safety cabinet and maintained in tissue culture flasks at 37°C in an atmosphere of 5% CO₂. Flasks contain 30–50 mL medium.
2. Cells are subcultured when confluent, as observed using an inverted microscope (every 2–3 days). Confluent monolayers are treated with trypsin/EDTA (4 mL) until the cells start to lift off the flask surface (2–5 min in the CO₂ incubator). The cells are resuspended in complete MEM (10 mL), centrifuged, and then resuspended in 5 mL complete MEM, before the cell concentration is measured by counting in a hemocytometer.
3. For adherence or confocal assays, a cell suspension is diluted to approximately 2×10^5 cells/mL in complete MEM, and 100 or 500 µL is seeded in wells of the 96- or 24-well plates, respectively (13 mm diameter sterile coverslips are first added to the 24-well plates) (*see* **Note 24**). The plates are incubated at 37°C in a 5% CO₂ atmosphere for 24 h to allow the formation of confluent monolayers, which are washed once with ASB (100 µL) before addition of ASB (50 or 250 µL to 96- or 24-well plates, respectively) and yeast cells (50 or 250 µL, respectively).

3.4.2. Adherence Assay Conditions

1. Washed radiolabeled *C. albicans* cells (2.2×10^6 cells/mL; 1.0 mL in microfuge tubes) are pre-treated with saliva (diluted 20–60% in ASB) at room temperature for 1 h with end-over-end mixing.
2. Cells are washed three times in ASB and 50 µL added to quadruplicate wells in 96-well microtiter plates containing

epithelial cell monolayers in 50 μL ASB (final volume of 100 μL per well, 1×10^6 yeast cells).

3. The plates are incubated at 37°C in an atmosphere of 5% CO_2 for 1.5 h. The liquid in the wells is then aspirated and the monolayers washed three times (*see* **Note 25**) with pre-warmed PBS (100 μL) before air-drying and addition of 100 μL Optiphase 3 scintillation fluid.
4. Determine bound radioactivity, and hence number of bound *C. albicans* cells, in each well by scintillation detection as above.

3.4.3. Confocal Microscopy

1. Yeast cells grown as for radiolabeling (but without addition of ^{35}S -methionine) are washed in water and resuspended (2.2×10^6 cells/mL; 10 mL) in carbonate/bicarbonate buffer pH 9.5 in a universal bottle wrapped in foil to exclude light.
2. Freshly weighed FITC powder (1 mg) is added and the cell suspension stirred for 1 h.
3. Cells are washed three times in ASB and 250 μL volumes incubated with epithelial cell monolayers in wells of 24-well plates at 37°C in an atmosphere of 5% CO_2 for 1.5 h.
4. The monolayers are washed three times with pre-warmed PBS and inverted onto a drop of PBS on a glass microscope slide for confocal microscopy (plates and slides are protected from light as much as possible). Confocal settings: FITC excitation is at 494 nm and emission is at 520 nm.
5. An example of *C. albicans* cells adhering to human epithelial cells is given in **Fig. 8.2**.

3.5. Adhesion of *C. albicans* or *S. epidermidis* Cells to Saliva- Coated Medical Grade Silicone or to Denture Prosthetic Materials

These assays that model the initial adherence of microbial cells to voice or dental prostheses use a similar approach for each of the materials used (adhesion under non-flow, static, conditions). Small rectangular slices (coupons) of medical grade silicone, or small molded pieces of denture prosthetic material, are incubated with radiolabeled yeast or bacterial cells in microfuge tubes. We pre-treat the materials with saliva or saliva wash to mimic in vivo conditions.

1. Individual silicone or dental material coupons, prepared as described in **Section 2.5**, are incubated in triplicate in glass tubes (of a diameter such that both faces of the coupon are exposed and the coupon is submerged completely) with 1.0 mL of saliva (or saliva wash or control solutions) with gentle agitation at room temperature for 2 h.
2. Coupons are then washed three times with KCl buffer (1.0 mL) and transferred to a microfuge tube (again such that both faces of the coupons are exposed and submerged) containing 0.9 mL KCl.

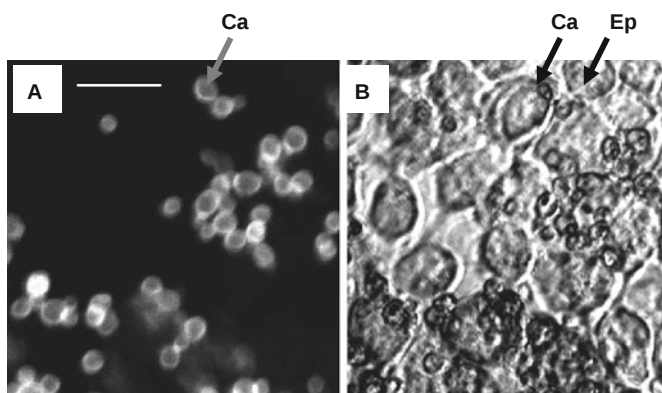


Fig. 8.2. Confocal microscopy (split field) showing adhesion of *C. albicans* ATCC 10261 yeast cells to a monolayer of cultured epithelial cells (HET1-A cell line kindly provided by Dr C.C. Harris, Laboratory of Human Carcinogenesis, NCI, NIH, Bethesda, MD). Yeast cells were labeled with FITC, washed, and incubated with confluent monolayers on glass coverslips in 24-well plates, before washing and mounting on glass slides for confocal microscopy. **a:** fluorescence micrograph showing bound yeast cells (bar = 25 μ m); **b:** same field of view by phase-contrast microscopy showing both epithelial (Ep) and *C. albicans* (Ca) cells.

3. To each tube, ^{35}S -methionine radiolabeled yeast cells (0.1 mL, 1.1×10^6 cells) or ^3H -thymidine radiolabeled bacterial cells are added (0.1 mL, 1.1×10^6 cells) and the tubes are incubated end over end at room temperature for 2 h (*see Note 26*).
4. Coupons are washed (1 mL KCl, $\times 3$) then transferred to a fresh microfuge tube containing 1.0 mL scintillant and radioactivity measured (*see Note 23*). An example of *C. albicans* adhesion to polymethyl methacrylate is given in Fig. 8.3.

3.6. Adhesion of *S. epidermidis* to Denture Prosthetic Materials Under Flow Conditions

3.6.1. Bacteria: (*S. epidermidis*)

Parallel plate flow chambers are used in dynamic studies of cell adhesion under well-defined shear forces (7) and the apparatus was adapted for the investigation of adherence of bacteria to the denture materials listed in Section 2.5. These materials were individually constructed as there is no commercial source for pre-made materials in the dimensions required.

Bacteria are grown in BHY (50 mL in 50 mL bottle) without added radiolabel at 37°C for 18 h and washed twice in FAB before resuspending cells to a concentration of 3×10^8 cells/mL, prior to circulation through the flow chamber.

3.6.2. Preparation of Denture Prosthetic Material Surfaces

A thin film of each material is manufactured and attached to a glass microscope slide using a gypsum mold and the denture flasking and pressing method (14).

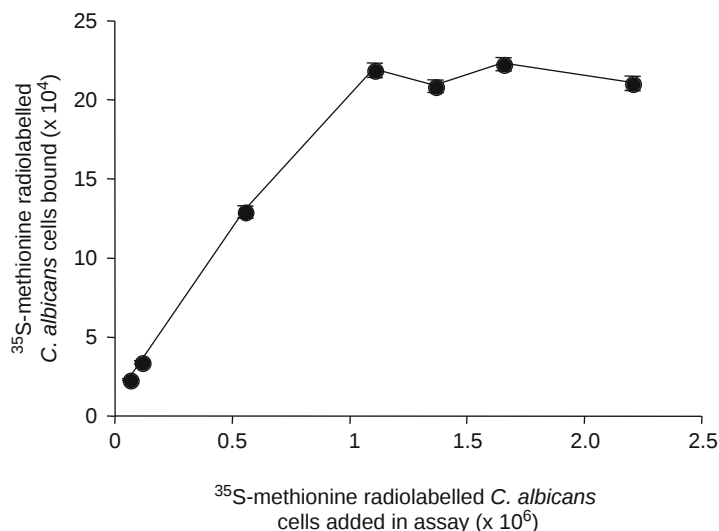


Fig. 8.3. Adhesion of ^{35}S -methionine-radiolabeled *C. albicans* ATCC 10261 cells to dental acrylic (Rimseal, polymethyl methacrylate) showing saturation of binding sites with an increasing number of cells added to the assay. Radiolabeled cells, 1.0 mL at different concentrations in KCl, were incubated end over end with coupons of dental acrylic that had been pre-treated with saliva (50% in KCl buffer for 2 h) in microfuge tubes at room temperature for 2 h. Coupons were washed and placed in fresh tubes containing scintillant before determination of coupon-associated radioactivity by scintillation counting. The numbers of cells bound were calculated from a standard curve. Results shown are the means $\pm$ SE of quadruplicate determinations from a representative experiment repeated twice.

1. The 75 mm $\times$ 25 mm $\times$ 1 mm glass slides are flaked in denture flasks in gypsum. In the lower denture flask, a microscope slide is placed flat and embedded in the gypsum.
2. Once the gypsum is set a second slide is placed on top of the embedded slide and stuck down with a small amount of wax. A thin layer of vaseline is applied to the surface of the stone to allow easy separation of the flasks.
3. The denture flask is then topped with yellow stone and left to set. Once the stone is set, the flasks are separated, giving flask halves with microscope slides embedded in the lower and opposing upper segments of the flasks which are the gently scrubbed with dishwashing liquid and boiling water to clean the microscope slides and remove the vaseline.
4. The glass slide in the lower flask is removed and replaced with a glass microscope slide measuring 75 mm $\times$ 25 mm $\times$ 0.8 mm. The slides are then dried and separating fluid applied to the stone surface surrounding the microscope slides for easy separation after processing.

3.6.3. Parallel Plate Flow Chamber Setup

1. The parallel plate flow chamber is mounted on the microscope stage.

2. Prior to adhesion testing, the parallel plate flow chamber plates are cleaned with a commercially available disinfectant (Virkon, DuPont, Wilmington, DE, USA), rinsed thoroughly with water, then ethanol, and finally demineralized water.
3. The glass plate and the denture prosthetic material plate are placed in the flow chamber.
4. Laminar fluid flow is achieved in the middle of the flow chamber by the slope of the inlet and outlet channels of the flow chamber.
5. With this system it is possible to directly monitor with a microscope, in real time, the initial adhesion of bacteria to the bottom, denture material, plate in a field of view of 0.5 mm.

3.6.4. Bacterial Deposition

1. Initially, FAB is re-circulated using a dual-channel variable-speed pump which re-circulates the bacterial and/or yeast suspension through 0.89 mm diameter tubing at a rate of 0.9 mL/min for 15 min.
2. FAB containing the bacterial suspension is then re-circulated through the flow chamber for 2 h enabling bacteria to adhere to the denture material. Photographic images of the center region of the substratum are taken every minute to measure adherence to the denture material. An example of *S. epidermidis* adhesion to polymethyl methacrylate with time is shown in **Fig. 8.4**.
3. After 2 h, flow is switched to buffer only for 30 min to remove non-adhering bacteria, with continued photography each minute. The chamber is then drained thus passing an air-liquid interface over the substratum surface and the adhering microorganisms.
4. Pre- and post-drainage images are compared to determine the number of bacteria that are detached by the surface tension force resulting from the passage of an air-liquid interface.
5. All experiments are performed at room temperature in triplicate with separate bacterial cultures.
6. To determine the influence of saliva on the rate of adhesion, the denture prosthetic material slide is incubated in clarified whole saliva (see Step 4 of **Section 2.3**) diluted 50% in KCl buffer for 2 h prior to mounting in the flow chamber.
7. The number of microorganisms adhering to the bottom plate of the flow chamber in the digital photographs is measured using image analysis software. The images of the adhering bacteria are discriminated from the substratum background by a single grey-value threshold yielding

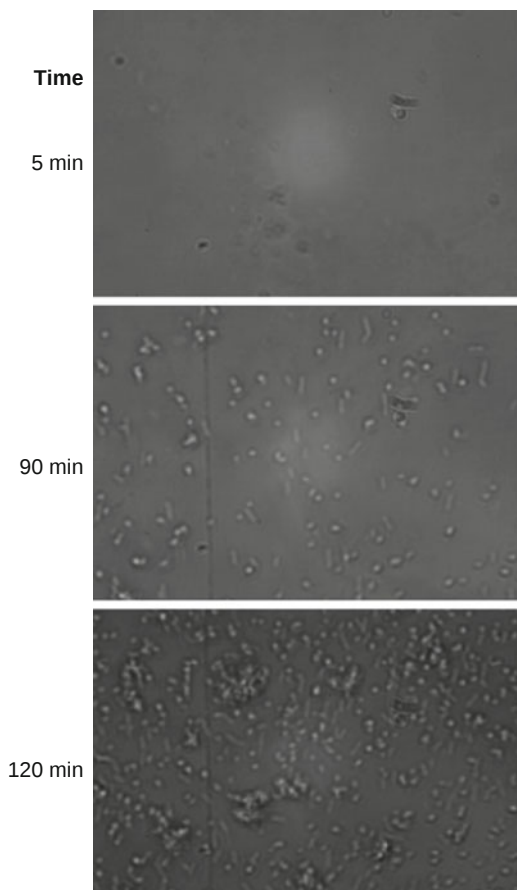


Fig. 8.4. Images showing the adhesion over time (three time points shown) of *S. epidermidis* cells to dental acrylic (heat-processed polymethyl methacrylate) in a parallel plate flow chamber.

a binary black and white image and the number of adhering bacteria in each image is counted using the software. The deposition rate is calculated based on the number of microorganisms adhering per unit time and area.

8. Control experiments are conducted with bacteria adhering to bare glass. All adhesion experiments are done in triplicate with freshly cultured bacteria.

4. Notes

1. Scintillation counter: we use a 1450 MicroBeta TriLux Scintillation counter (LKB, Wallac Oy, Turku, Finland).
2. Safe handling of radioactive materials is very important. The radioactive isotopes used in the assays described

(^{35}S and ^3H) do not produce penetrating radiation and can be handled in a normal laboratory as long as the appropriate regulatory body requirements are met. The principal hazard is ingestion and therefore avoidance of skin contact is the most important safety step – use double gloves and appropriate personal protective equipment including gown and face shield. When first opening a vial of ^{35}S -methionine open it in a fume hood in case there is some release of volatile radioactive material.

3. Incorporation of the radiolabel into microbial cells: we have conducted experiments that have shown that the incorporation of radiolabeled methionine or thymidine into yeast or bacterial cells, respectively, is stable under the experimental conditions used and that negligible radiolabel is lost from cells during the experiments. The specific radioactivity of the cells can be calculated by measuring the radioactivity of known numbers of cells. In our experiments the radiolabeled yeast cells had a mean activity of 32 ± 18 cells/cpm and 18 ± 7 cells/cpm for bacteria.
4. GSB medium can be conveniently made up from concentrated stocks of solutions stored at 4°C . For example, a $5\times$ stock of the salts solution and a $1,000\times$ stock of biotin can be diluted as required and glucose added at the required concentration before autoclaving in a suitable culture or storage vessel. Although biotin is a vitamin and can be unstable at high temperatures, sufficient functional biotin is retained after autoclaving to support yeast growth.
5. The equipment required for electrophoresis and blotting is commercially available; for electrophoresis we use a mini-PROTEAN apparatus (Bio-Rad) and for electroblotting a Mini Trans-Blot Cell (Bio-Rad). Direct current is supplied by a PowerPac Basic Power Supply (Bio-Rad).
6. Acrylamides are neurotoxins – handle with care. Purchase ready-made solutions rather than making stock solutions to avoid weighing solid acrylamide. Wear gloves for all stages of gel preparation.
7. Ammonium persulfate (10% w/v) keeps for several weeks at 4°C . Best practice is to store it frozen in small aliquots (approx 0.5 mL) which can be thawed as required.
8. Pre-stained markers are useful for checking success of protein transfer during electroblotting and also for orientation of blots following development of the overlay autoradiograph.
9. Transfer buffer: always dissolve the Tris and glycine in water (approximately 600 mL) before adding the methanol and

making up to volume (adding methanol first gives an insoluble precipitate).

10. Confocal microscope: We used a Bio-Rad MRC-600 (Bio-Rad Microscience Ltd, Hemel Hempstead, UK) with a krypton/argon laser (which produced excitation lines at 488, 568, and 647 nm) and a Nikon Diaphot inverted microscope (Nikon Instruments, Inc., Melville, NY).
11. Carbonate/bicarbonate buffer: prepare as two solutions (each of 0.1 M) – can be mixed in different ratios to give a range of pH values.
12. Place silicone slice on clear smooth surface (e.g., plastic Petri dish) over a ruled template so that the correct size can be cut.
13. Saliva wash samples are used rather than saliva for individuals (for example, voice prosthesis patients who have had head and neck radiotherapy) with poor saliva flow and/or saliva with high mucin content. Such “wash” samples are much simpler to handle in the laboratory and we have still been able to detect in them saliva proteins involved in adherence.
14. Parallel plate flow chamber: the parallel plate flow chamber is commercially available (Glycotech, Rockville, MA, USA) and is constructed from stainless steel (dimensions: $l \times w \times h = 200 \text{ mm} \times 42 \text{ mm} \times 10 \text{ mm}$) and contains two glass plates (dimensions $l \times w \times h = 76 \text{ mm} \times 26 \text{ mm} \times 1 \text{ mm}$), separated by 0.6 mm using a Teflon spacer, that constitute the top and bottom plates of the chamber.
15. We used an Olympus IMT-2 inverted phase contrast microscope, Olympus Corporation, Tokyo, Japan, with an ultra-long working distance 40 $\times$ objective (Olympus ULWD-CD Plan 40) for observation of bacteria, and a 10 $\times$ objective (Olympus SPlan 10) to observe yeast.
16. We use a Shimadzu spectrophotometer at OD₅₄₀ for yeast cells and OD₆₀₀ for bacterial cells.
17. We construct a standard curve relating OD₅₄₀ values to *C. albicans* cells per milliliter by measuring the OD₅₄₀ with a spectrophotometer and measuring the cell concentration for an appropriate dilution of the same culture with a hemocytometer.
18. The half-life of ³⁵S is approximately 3 months. With preparations of methionine that have been stored for periods longer than a few weeks, the amount of radiolabel added can be increased to 4 or 5 μL .
19. We have used the same technique to demonstrate adhesion of *C. albicans* to various proteins, including

recombinant proteins that have been cloned and expressed in *Escherichia coli*. The technique was first developed to demonstrate the adherence of *C. albicans* to salivary proline-rich proteins (9).

20. Total protein is estimated using a Bradford protein assay kit (Bio-Rad).
21. Destaining Coomassie-stained PAGE gels: a small pad of sponge material (for example, from packing material) added to the destain with the gel helps to remove background stain from the gel.
22. Care must be taken when electroblotting small proteins as they may transfer rapidly and can be lost through the nitrocellulose if electroblotting is continued for too long. Conversely large proteins may not transfer well and require prolonged electroblotting.
23. Static electricity may be generated while handling the microfuge tubes with latex gloves and this may give a false scintillation counter reading. Take a second reading 24 h after the initial reading to allow static electricity to discharge.
24. To avoid “cross-talk” in the scintillation counter, leave some wells unfilled: use only alternate columns of wells and alternate rows of wells.
25. Aspirating and washing tissue culture monolayers: aspirate from the edge of the monolayer gently with manual multi-channel pipette. Also, add wash solution gently down side of well wall – reverse plate 180° for alternate washes. It is important to pre-warm the wash solution to 37°C to prevent loss of tissue culture cells.
26. Ensure that the silicone coupon is completely submerged in the solution containing the radiolabeled cells for the entire duration of incubation to allow cell adhesion to the entire surface of the silicone coupons. Also check that the liquid moves up to the top of the tube and back during each rotation. If not (this is possibly a surface tension effect) stop rotation, remove and invert the tubes a few times, replace, and restart. Repeat if necessary.

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References

1. Jenkinson, H. F., and Lamont, R. J. (2005) Oral microbial communities in sickness and in health. *Trends Microbiol.* **13**, 589–595.
2. Kolenbrander, P. E. (2000) Oral microbial communities: biofilms, interactions, and genetic systems. *Ann. Rev. Microbiol.* **54**, 413–437.
3. Moscona, A. (2008) Medical management of influenza infection. *Ann. Rev. Med.* **59**, 397–413.
4. Sharon, N. (2006) Carbohydrates as future anti-adhesion drugs for infectious diseases. *Biochim. Biophys. Acta.* **1760**, 527–537.
5. Cannon, R. D., Holmes, A. R., Mason, A. B., and Monk, B. C. (1995) Oral *Candida*: clearance, colonization, or candidiasis? *J. Dent. Res.* **74**, 1152–1161.
6. Meurman, J. H. (2005) Probiotics: do they have a role in oral medicine and dentistry? *Eur. J. Oral Sci.* **113**, 188–196.
7. Busscher, H. J., and van der Mei, H. C. (2006) Microbial adhesion in flow displacement systems. *Clin. Microbiol. Rev.* **19**, 127–141.
8. Cannon, R. D., Nand, A. K., and Jenkinson, H. F. (1995) Adherence of *Candida albicans* to human salivary components adsorbed to hydroxyapatite. *Microbiology.* **141**, 213–219.
9. O'Sullivan, J. M., Cannon, R. D., Sullivan, P. A., and Jenkinson, H. F. (1997) Identification of salivary basic proline-rich proteins as receptors for *Candida albicans* adhesion. *Microbiology.* **143**, 341–348.
10. O'Sullivan, J. M., Jenkinson, H. F., and Cannon, R. D. (2000) Adhesion of *Candida albicans* to oral streptococci is promoted by selective adsorption of salivary proteins to the streptococcal cell surface. *Microbiology.* **146**, 41–48.
11. Holmes, A. R., Bandara, B. M., and Cannon, R. D. (2002) Saliva promotes *Candida albicans* adherence to human epithelial cells. *J. Dent. Res.* **81**, 28–32.
12. Holmes, A. R., van der Wielen, P., Cannon, R. D., Ruske, D., and Dawes, P. (2006) *Candida albicans* binds to saliva proteins selectively adsorbed to silicone. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **102**, 488–494.
13. Dawes, C. (2008) Salivary flow patterns and the health of hard and soft oral tissues. *J. Am. Dent. Assoc.* **139**(Suppl), 18S–24S.
14. Morrow, R. M., Rudd, K. D., and Rhoads, J. E. (1986) *Dental laboratory techniques – complete dentures*. Mosby, St Louis, pp. 287–311.

Chapter 9

Quantitative Analysis of Periodontal Pathogens by ELISA and Real-Time Polymerase Chain Reaction

Stephen M. Hamlet

Abstract

The development of analytical methods enabling the accurate identification and enumeration of bacterial species colonizing the oral cavity has led to the identification of a small number of bacterial pathogens that are major factors in the etiology of periodontal disease. Further, these methods also underpin more recent epidemiological analyses of the impact of periodontal disease on general health. Given the complex milieu of over 700 species of microorganisms known to exist within the complex biofilms found in the oral cavity, the identification and enumeration of oral periodontopathogens has not been an easy task. In recent years however, some of the intrinsic limitations of the more traditional microbiological analyses previously used have been overcome with the advent of immunological and molecular analytical methods. Of the plethora of methodologies reported in the literature, the enzyme-linked immunosorbent assay (ELISA), which combines the specificity of antibody with the sensitivity of simple enzyme assays and the polymerase chain reaction (PCR), has been widely utilized in both laboratory and clinical applications. Although conventional PCR does not allow quantitation of the target organism, real-time PCR (rtPCR) has the ability to detect amplicons as they accumulate in “real time” allowing subsequent quantitation. These methods enable the accurate quantitation of as few as 10^2 (using rtPCR) to 10^4 (using ELISA) periodontopathogens in dental plaque samples.

Key words: Polymerase chain reaction (PCR), real-time PCR (rtPCR), enzyme-linked immunosorbent assay (ELISA), periodontitis, periodontal disease, oral periodontopathogens.

1. Introduction

The key role of bacteria in the etiology of periodontal disease has been well established (1, 2). Putative pathogens such as *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, and *Treponema*

denticola have been identified from association studies, the demonstration of virulence properties, and the demonstration of antibody responses in patients (3). Given the milieu of microorganisms that normally reside in the oral cavity, the accurate identification and enumeration of these putative pathogens is no minor task. Gmür and Lüthi-Schaller (4) have suggested that the oral cavity is normally colonized by a commensal microflora of more than 10^{11} microorganisms forming numerous complex biofilms. Further, the identification and enumeration of oral periodontopathogens is often complicated by the presence of bacteria with unusual phenotypic profiles, rare bacteria, slow growing, or often uncultivable bacteria. With the advent of new molecular techniques, rather than simplifying this problem, the discovery of novel species is as yet unabated. Woo et al. (5) using 16S rDNA sequencing have identified 215 novel bacterial species in human specimens, 29 of which belong to novel genera, just within the last 7 years. Importantly, these authors also demonstrated that the oral cavity is one of the most important sites for the discovery of and/or reservoirs of novel species (5).

Typically the identification and enumeration of bacteria has relied on three main types of analysis: phenotypic, serological, and genetic. Phenotypic methods are relatively inexpensive and easy to perform. However, their biggest drawback has always been the need to culture the organism of interest. Other problems often include (i) the fastidious requirements for sample collection when dealing with anaerobes, (ii) slow growing or non-cultivable organisms, and (iii) identification tests that are not always definitive or quantitative.

Serological tests are designed to take advantage of the specific nature of antibodies. The normal polyclonal immune response to the presence of antigen, where a number of antibodies are produced which recognize different epitopes present on the antigen, is of limited use in clinical laboratory assays. For example, a common epitope present in different bacterial species would result in a non-specific assay. Any inconsistent immune response by different animals used during production of the antibody may also result in significant inter-assay variation. In the early 1970s, the development of techniques for producing monoclonal antibodies (mAb), i.e., one cell line producing the same antibody of known specificity, revolutionized the use of immunological methods in both clinical and laboratory research. Spleen cells from an animal previously vaccinated with the antigen under consideration are fused with myeloma cells to produce a "hybridoma." These immortalized antibody-producing cell lines are then screened for antigen specificity and usually conjugated to a reporter molecule (enzymatic/fluorescent). The resultant enzyme-linked immunosorbent assay (ELISA) combines the specificity of antibodies (particularly monoclonal antibody) with the sensitivity of simple enzyme

assays. While many variations for performing this assay have been developed and described in the literature, the basic steps have remained remarkably consistent as follows: (1) coat a solid support (most commonly microtiter plate wells) with antigen, (2) add specific antibody to the wells conjugated to an enzyme reporter molecule, and (3) react substrate with enzyme to produce a colored product indicating a positive reaction. The direct ELISA therefore affords a fast turnaround of samples – as only one antibody is used, and the potential for cross-reactivity of a secondary antibody is eliminated. However some disadvantages include the immunoreactivity of the primary antibody which may be reduced as a result of labeling, and there is little signal amplification limiting assay sensitivity.

To overcome some of these limitations, the indirect or “sandwich ELISA” has been the most widely used variation. In this method, two antibodies are utilized as follows: (1) coat a solid support (most commonly microtiter plate wells) with antigen, (2) add specific antibody to the wells, (3) add a second non-specific antibody conjugated to an enzyme that binds IgG, and (4) react substrate with the enzyme to produce colored product indicating a positive reaction. This assay now allows the use of a wide variety of commercially available labeled secondary antibodies, immunoreactivity of the primary antibody is not affected by labeling, and sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody. Again, some disadvantages of indirect detection may include cross-reactivity occurring with the secondary antibody resulting in non-specific signal and an extra incubation step is required in the procedure.

Genetic methods are broadly described as those based on the DNA sequence of the target organism and currently include hybridization to complementary DNA sequences (DNA probes) using the checkerboard platform (6), fluorescence in situ hybridization (FISH) with labeled oligonucleotide probes (4), polymerase chain reaction (PCR), in particular real-time PCR using species (7) or clone-specific primers (8), and sequencing of libraries of cloned PCR amplified 16S rRNA genes (5, 9). The polymerase chain reaction developed in the mid-1980s, would arguably be the most utilized technique for the detection and enumeration of oral pathogens today. PCR owes its success to the discovery of the properties of the thermostable DNA polymerase from *Thermus aquaticus* (*Taq*), which has allowed the amplification of small quantities of DNA into useful amounts. Although conventional PCR does not allow precise quantitation of the target organism, for completeness this chapter does include a method describing the qualitative detection of *T. forsythensis* in dental plaque using conventional PCR. Quantitation, however, is readily achievable using real-time PCR (rtPCR).

rtPCR has the ability to detect PCR products as they accumulate in “real time” rather than estimating the amount of target accumulated after a fixed number of cycles (as in conventional PCR). The TaqMan[®] process described in this chapter achieves this by utilizing a fluorogenic probe designed to anneal to a specific sequence of template between the sense and anti-sense primers. This oligonucleotide probe is constructed to contain a reporter fluorescent dye at the 5′ end and a quencher dye at the 3′ end that disrupts any observable signal from the reporter dye when it is within a short distance of the reporter dye. Subsequent cleavage of the probe by the 5′-exonuclease activity of the DNA polymerase during the extension phase of the PCR process separates the reporter dye from the quencher dye, increasing the reporter dye signal. Alternatively, the SYBR[®] Green chemistry uses SYBR[®] Green I dye, a highly specific double-stranded DNA binding dye which detects amplification product as it accumulates during each PCR cycle. In both cases, the rtPCR is characterized by the point in time during cycling at which amplification of the target is first detected rather than the end point in conventional PCR. Hence, the higher the starting copy number of the nucleic acid target, the sooner (i.e., at a lower cycle number) a significant increase in fluorescence is detected. Therefore, compared to conventional PCR, rtPCR has the following advantages: (i) data is collected in the exponential growth phase with the increase in reporter fluorescent signal being directly proportional to the number of amplicons generated allowing quantitation, (ii) increased dynamic range of detection, and (ii) no-post PCR processing.

In this chapter, the methodology for the detection and/or quantification of specific oral pathogens in dental plaque using both direct and sandwich ELISA techniques is provided. Clearly, these methods are suitable for any oral organism if the laboratory has access to the relevant specific monoclonal antibody. Similarly, as more oral bacterial genomes are sequenced and become available for detailed examination, it will only require straightforward modification of the following PCR assay protocols to enable their quantitation.

2. Materials

2.1. Sample Collection

1. Phosphate buffered saline (PBS) pH 7.3. 20× stock PBS: 320 g NaCl, 8 g KH₂PO₄, 46 g Na₂HPO₄, 8 g KCl. Make up to 2 L with distilled H₂O, adjust to pH 7.3. Store at room temperature (RT).

2. Sterile glass beads (~1 mm diameters).
3. Thimerosal (Sigma Aldrich, Australia).
4. 0.2 μm Medikap2 filter.
5. 2 mL cryovials (Nalgene, USA).
6. 1 L sterile Schott bottles.
7. Sterile curettes.
8. Vacutainers (no anticoagulant).

2.2. Direct ELISA (Dental Plaque)

1. Bacterial suspensions of known concentration (cells/mL) in PBS pH 7.3: *A. actinomycetemcomitans*, *P. gingivalis*, and *Prevotella intermedia*. Store at -20°C (see **Note 1**).
2. Horse radish peroxidase labeled monoclonal antibody (HRP-mAb) (see **Note 2**).
3. 0.1 M carbonate buffer (pH 9.6): per 1 L: 10.6 g Na_2CO_3 , 8.4 g NaHCO_3 , 0.2 g NaN_3 . Store at 4°C .
4. 100 mM phosphate citrate buffer (pH 3.5): per L: 100 mM Na_2HPO_4 , 0.05 mM EDTA. Add 100 mM citric acid until desired pH 3.5 is reached. Store at 4°C .
5. PBS pH 7.3 (see **Section 2.1**).
6. PBS–0.05% Tween 20, pH 7.3. Store at RT.
7. 1% Newborn calf serum. Store at -20°C .
8. 0.025 M *o*-tolidine (Eastman Kodak, USA). Store at 4°C .
9. 3% H_2O_2 . Store at 4°C .
10. 3% HCl. Store at 4°C .
11. Ultrasonicator (Sonics & Materials, USA).
12. “Maxisorp” 96-well microtiter plate (Nunc, Denmark).
13. Microplate reader.

2.3. Pathogen-Specific Serum IgG ELISA

1. Bacterial suspensions of known protein concentration ($\mu\text{g/mL}$) in PBS pH 7.3: *A. actinomycetemcomitans*, *P. gingivalis*, and *Pr. intermedia*. Store at -20°C .
2. Horse radish peroxidase (HRP) labeled rabbit-anti-human IgG (DakoCytomation, Australia). Store at 4°C .
3. Human IgG (Zymed, USA). Aliquot in PBS pH 7.3. Store at -20°C .
4. 0.1 M carbonate buffer pH 9.6 (see **Section 2.2**).
5. PBS–0.05% Tween 20, pH 7.3 (see **Section 2.2**).
6. 1% Bovine serum albumin (BSA). Store at -20°C .
7. 0.025 M *o*-tolidine (see **Section 2.2**).
8. 3% H_2O_2 (see **Section 2.2**).

9. 3% HCl (*see* **Section 2.2**).
10. Phosphate citrate buffer pH 3.5 (*see* **Section 2.2**).
11. BCATM Protein Assay (Pierce, USA).

2.4. DNA Extraction

No specialized equipment required other than general laboratory equipment and reagents previously mentioned (*see* **Note 3**).

2.5. Conventional PCR (*T. forsythensis*)

1. PBS: 20× stock solution pH 7.3 (*see* **Section 2.1**) (*see* **Note 3**).
2. Primers (18 μM each): sense (5'-AAAACAGGGGTTCCGCATGG-3') and antisense (5'-TTCACCGCGGACTT-AACAGC-3') (Sigma Aldrich, Australia). Store at -20°C (*see* **Notes 4–6**).
3. 0.2 mL thin-walled PCR reaction tubes (Quality Scientific Plastics, USA).
4. 20 mM deoxyribonucleic acid (dNTP) mixture.
5. AmpliTaq Gold[®] kit containing 5 U/μL AmpliTaq Gold[®] DNA Polymerase, 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), and 25 mM MgCl₂ (Applied Biosystems, Australia). Store at -20°C.
6. Programmable thermal cycler.

2.6. Agarose Gel Electrophoresis

1. Tris-acetate (TAE) buffer, 20× stock: 193.84 g Tris base, 14.89 g EDTA, adjust to pH 8.0 with glacial acetic acid. Make up to 2 L with distilled H₂O. Store at RT.
2. 2% PCR agarose in TAE buffer. Store at RT.
3. 10 mg/mL ethidium bromide (EtBr). Store at RT.

2.7. rtPCR (*P. gingivalis* and *Fusobacterium nucleatum*)

1. Suspension of 10⁹ cells (*P. gingivalis* and *F. nucleatum*) in PBS pH 7.3 (*see* **Note 7**).
2. ABsoluteTM QPCR Mix containing Thermo-Start DNA polymerase, proprietary reaction buffer, ROX reference dye, and dNTPs (ABgene, UK) (*see* **Note 8**).
3. 96-well plates – Thermofast 0.2 mL thin-walled non-skirted (ABgene, UK).
4. ABI Prism 7700 thermocycler (Applied Biosystems, Australia).
5. Fluorogenic Probes (5 μM): *P. gingivalis* (5'-(6-Fam)-AACGAGCGCAACCCACATCGGT-(Tamra)-3'); *F. nucleatum* (5'-(6-Fam)-TCGACGCAACGCGAGGAACCTT-(Tamra)-3') (Sigma Aldrich, Australia). Store at -20°C (*see* **Notes 6 and 9**).
6. Primers (18 μM each): *P. gingivalis* sense: 5'-GGT-GTCGGCTTAAGTGCCA-3'; antisense: 5'-CCTCAGCG-

AAAACTGTTAGCAA-3'; *F. nucleatum* sense: 5'-CGGTG-GAGCATGTGGTTTAA-3'; antisense: 5'-TTCCTAAGAT-GTCAAACGCTGG-3' (Sigma Aldrich, Australia). Store at -20°C (*see* **Notes 5, 6, 10**).

7. BCA™ Protein Assay (Pierce, USA) (*see* **Note 11**).

3. Methods

3.1. Sample Collection

1. Filter sterilize 2 L of PBS-0.01% thimerosal using a 0.2 µm Medikap2 filter with filling bell into 1 L sterile Schott bottles.
2. In a biosafety cabinet, add approximately 10 sterile glass beads to each Nalgene cryovial and 1.5 mL of the filter-sterilized PBS-0.01% thimerosal solution.
3. Sealed vials may be kept indefinitely in a cool dark storage place at RT.
4. Subgingival plaque samples (*see* **Note 12**) are collected with a sterile curette (*see* **Note 13**) after any supragingival plaque has been removed and placed into the sterile PBS-0.01% thimerosal solution.
5. Store vials containing sample at 4°C. For long-term storage, store samples at -80°C (*see* **Note 14**).

3.2. Direct ELISA (Dental Plaque)

1. Thaw samples and disperse plaque by vortexing briefly and then sonicating for 5 s.
2. The suspension is then further diluted in an equal volume of 0.1 M carbonate buffer before transferring 100 µL aliquots into wells of a "Maxisorp" microtiter plate (*see* **Note 15**).
3. To allow quantification of bacterial numbers, 100 µL aliquots of known concentrations of either *A. actinomycetemcomitans* (FDC Y4), *P. gingivalis* (FDC 381), or *Pr. intermedia* (ATCC 25611) ranging from 150×10^4 to 9×10^4 cells/mL in carbonate buffer are also transferred to the plate (*see* **Notes 1 and 16**).
4. The plates are then incubated overnight at 4°C in a moist plastic bag.
5. Wash plates ($\times 3$) in PBS-0.05% Tween20 pH 7.3.
6. Non-specific binding is then blocked by incubating plates for 1 h at RT with PBS-0.05% Tween 20 containing 1% newborn calf serum.

7. Following washing ($\times 3$) in PBS–0.05% Tween20, diluted HRP-mAb, specific for the pathogen being detected, is added to the coated wells and incubated for 2 h at RT (*see Note 2*).
8. Wash plates ($\times 3$) in PBS–0.05% Tween 20.
9. Add 150 μL per well freshly prepared color substrate (per plate – 15 mL phosphate citrate buffer pH 3.5, 15 μL 3% H_2O_2 , 1.5 mL 0.025 M *o*-toluidine).
10. The resulting reaction is stopped after 10–15 min by adding 50 μL of 1 M HCl.
11. Plates are then read in a microplate reader at 450 and 655 nm.

3.3.

Pathogen-Specific Serum IgG ELISA

1. Collect 5 mL of blood from an antecubital vein into a plain vacutainer.
2. Separate serum by centrifugation (500*g* for 10 min) at 4°C (*see Note 17*).
3. Aliquot serum into labeled Nalgene cryovials.
4. Store serum samples at –20°C. For long-term storage, store samples at –80°C.
5. Add 100 μL /well of diluted standard IgG (highest concentration standard = 500 ng/mL) (*see Note 16*) to appropriate wells (*see Table 9.1*).

Table 9.1

Pathogen-specific serum IgG ELISA example 96-well microtiter plate setup for the first overnight incubation. Std1–7 represents a 1 in 2 serial dilution curves of human IgG starting at 500 ng/mL. Ag represents the bacterial suspension at 1 $\mu\text{g/mL}$. CB represents the 0.1 M carbonate buffer pH 9.6 (these wells will ultimately act as negative controls for each individual serum sample)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std1	Std1	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB
B	Std2	Std2	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB
C	Std3	Std3	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB
D	Std4	Std4	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB
E	Std5	Std5	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB
F	Std6	Std6	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB
G	Std7	Std7	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB
H	Ag	Ag	Ag	Ag	Ag	CB	CB	Ag	Ag	Ag	CB	CB

6. Add 100 μL /well of 1 $\mu\text{g}/\text{mL}$ bacterial suspension to appropriate wells (determine the protein concentration of the bacterial suspension using a BCATM protein assay).
7. Add 100 μL /well of 0.1 M carbonate buffer pH 9.6 to negative control wells.
8. Plates are incubated overnight at 4°C in a sealed moist plastic bag.
9. Wash plates ($\times 3$) with PBS–0.05% Tween 20.
10. Block plates with 300 μL /well of 1% BSA solution and incubate at RT for 1 h.
11. Wash plates ($\times 3$) with PBS–0.05% Tween 20.
12. Add 100 μL /well of serum (diluted in PBS pH7.3) to the appropriate wells (5 wells per plate) and incubate at RT for 2 h. Serum dilutions are usually in the range of 1/50 to 1/100, but may need to be altered if results obtained are outside the range of standard curve (*see* **Note 18** and **Table 9.2**).
13. Wash plates ($\times 3$) with PBS–0.05% Tween 20.
14. Add 100 μL /well of diluted antibody (1/5,000 in PBS–0.05% Tween 20) to each well and incubate at RT for 1 h.
15. Wash plates ($\times 3$) with PBS–0.05% Tween 20.
16. Add 150 μL per well freshly prepared colorimetric substrate (*see* **Section 3.2**).

Table 9.2

During the 2-h serum incubation period, PBS is added to the wells containing the standard IgG to prevent the wells from drying out. Dilutions for the unknown samples may need to be re-evaluated depending on the results obtained. In our laboratory, however, the standard curve and dilutions used here were found to give the highest sensitivity for quantitation

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS only		Sample 1 diluted 1:50 in PBS					Sample 1 diluted 1:100 in PBS				
B	PBS only		Sample 2 diluted 1:50 in PBS					Sample 2 diluted 1:100 in PBS				
C	PBS only		Sample 3 diluted 1:50 in PBS					Sample 3 diluted 1:100 in PBS				
D	PBS only		Sample 4 diluted 1:50 in PBS					Sample 4 diluted 1:100 in PBS				
E	PBS only		Sample 5 diluted 1:50 in PBS					Sample 5 diluted 1:100 in PBS				
F	PBS only		Sample 6 diluted 1:50 in PBS					Sample 6 diluted 1:100 in PBS				
G	PBS only		Sample 7 diluted 1:50 in PBS					Sample 7 diluted 1:100 in PBS				
H	PBS only		Sample 8 diluted 1:50 in PBS					Sample 8 diluted 1:100 in PBS				

17. Incubate 10–15 min to ensure good color development of standards and samples.
18. Stop the color reaction with 50 μL per well of 3% HCl (*see Section 3.2*).
19. Read plate at 450 and 655 nm (*see Section 3.2*).

3.4. DNA Extraction

1. Vortex plaque sample/bacterial standard (for rtPCR) for 15 s to disperse cells (*see Note 11*).
2. Aliquot 0.5 mL of the plaque suspension or 10^9 bacterial cells (*P. gingivalis* and *E. nucleatum*) for rtPCR standard curve into a clean DNase-free Eppendorf tube and centrifuge at 10,000*g* for 10 min.
3. Wash the pellet and resuspend in 100 μL sterile DNase-free H_2O (*see Note 6*).
4. Puncture the lid of the Eppendorf tube and in a water bath boil the suspension for 20 min (*see Note 19*).
5. Place immediately on ice.
6. After 10 min, centrifuge at 10,000*g* for 2 min to remove cellular debris.
7. The supernatant containing the DNA is collected and stored frozen (-80°C) until analysis.
8. Prepare a seven-step 10-fold serial dilution in DNase-free H_2O of the DNA extracted from the bacterial standards (standards are equivalent to 10^9 – 10^2 cells) (*see Note 20*).

3.5. Conventional PCR (*T. forsythensis*)

1. Aliquot 45 μL of PCR master mix (30.5 μL DNase-free H_2O , 5 μL 10 $\times$ buffer, 2 μL dNTPs, 5 μL MgCl_2 , 1 μL each primer (18 pmol/ μL), and 0.5 μL *Taq* polymerase) into a 0.2 mL thin-walled PCR reaction tube in a DNA-free environment (*see Notes 3 and 21*).
2. Add 5 μL of extracted DNA (*see Section 3.4*) from specimens to the PCR tubes.
3. Place tubes in thermocycler and run the following amplification protocol: initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 30 s (denaturation), 60°C for 1 min (primer annealing), and 72°C for 1 min (extension). A final extension step at 72°C for 2 min completes the cycling program.
4. Analyze PCR aliquots (10 μL) by agarose gel electrophoresis.

3.6. Gel Electrophoresis

1. Clean minigel tray (BioRad Laboratories Inc.) and tape ends.
2. Melt 2% agarose (in TAE buffer) in microwave and cool to 60°C .

3. In a 50 mL Falcon tube, add 2 mL of 20× TAE buffer, 2 μ L EtBr, 40 mL of cooled agarose solution (*see* **Notes 22** and **23**).
4. Mix gently and pour into minigel tray, remove any bubbles, insert combs, and allow gel to solidify (*see* **Note 24**).
5. Remove combs, place in gel tank, load samples (10 μ L/well), and run at 60 V for $\sim$ 1 h (*see* **Note 25**).
6. The expected single 426-base pair PCR product is visualized by UV transillumination.

3.7. Real-time PCR (*P. gingivalis* and *F. nucleatum*)

1. Add 20 μ L of PCR master mix (5.16 μ L DNase-free H₂O, 12.5 μ L qPCR mix, 2 μ L dNTPs, 0.42 μ L each primer, 1.5 μ L probe, 0.375 μ L ROX, and 0.5 μ L *Taq* polymerase) to a 0.2 mL thin-walled PCR reaction tube in a DNA-free environment (*see* **Notes 3, 21, and 26**).
2. Add 5 μ L of extracted DNA (*see* **Section 3.4**) from specimens into the 0.2 mL PCR tubes (*see* **Note 20**).
3. Seal plate with flat cap strips (*see* **Note 27**).
4. Place tubes in thermocycler and run the following TaqMan[®] protocol: 15 min initial denaturation at 95°C followed by 40 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (extension) (*see* **Note 28**).
5. Analyze results with the ABI sequence detection system software (v1.9) (Applied Biosystems, Australia) (*see* **Note 29**).

4. Notes

1. The concentration (cells/mL) of the bacterial cell suspensions used for the construction of the standard curves in the direct ELISA may be determined using McFarland turbidity standards.
2. All pathogen-specific HRP-mAb was prepared and tested for sensitivity and specificity in-house (**10, 11**).
3. Due to the extreme sensitivity of PCR (ability to amplify only a few molecules of target DNA) extreme care must be taken to prevent “carryover” contamination due to the improper handling of samples; it only takes one molecule of amplicon to produce a false positive (**12**). To avoid contamination ensure the use of DNase/RNase free reagents and consumables, either certified or autoclaved before use, and filtered pipette tips for aliquoting solutions. DNase-free H₂O (*see* **Note 6**) was used for all reagents and master

mixes. Physical separation of Pre-PCR (master mix preparations) and Post-PCR (PCR and agarose gel electrophoresis) areas are ideal and best achieved by having one room dedicated to each process. These dedicated Pre- and Post-PCR rooms must also have dedicated equipments, such as pipettes and lab coats, to ensure activities at both stages remain completely separate, and nothing should leave the post-PCR room unless in a sealed waste container or soaked in 10% bleach for 4 h.

4. Primers were designed to the 16S ribosomal RNA sequence of *T. forsythensis* strain 338 (GenBank accession number L16495) with primer sequences corresponding to nucleotide positions 180–199 (sense) and 605–586 (anti-sense) (13).
5. To prevent contamination of primer stocks, commercially acquired stocks can be resuspended to a 200 μ M stock solution and diluted with DNase-free water to the required working concentration. Where more than one primer is used, both primers can be mixed in the same working solution. Stock solutions should be stored at -20°C and the working solutions at 4°C .
6. To prevent contamination or degradation of the DNA sample always use DNase-free H_2O . This can be produced by adding 500 μL diethylpyrocarbonate (DEPC) to 1 L deionized water (MilliQ H_2O ; resistivity of $18.2 \text{ M}\Omega/\text{cm}$), shake bottle thoroughly, let stand overnight in fume cupboard, and autoclave the next day.
7. The numbers of bacteria used for the construction of the standard curves for rtPCR were determined microscopically using a Petroff Hausser counting chamber.
8. The ABsoluteTM QPCR Mix should be stored at -20°C . A fresh 1:40 ROX working solution prepared by diluting in DNase-free H_2O is required before each rtPCR assay.
9. Maeda et al. (14) demonstrated that there is no significant difference between the TaqMan[®] and SYBR[®] Green chemistries in their specificity, quantitation, and sensitivity and thus SYBR[®] Green could be used as a more inexpensive alternative in this assay.
10. Oligonucleotide sequences were derived from the 16S rRNA gene. Primer Express software (version 1.5 [PE Applied Biosystems]) was used to design the TaqMan[®] probe and the sense and antisense primers. Probe and primers were assessed for species specificity using the Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/BLAST>).

11. The total protein concentration of the plaque sample is determined according to the manufacturer's instructions prior to extraction of the genomic DNA. Bacterial numbers determined in the PCR analysis may then be expressed per milligram of plaque protein to account for sampling differences.
12. This preservative medium has also been used successfully with gingival crevicular fluid collected with dental paper points.
13. Jervøe-Storm et al. (8) demonstrated by rtPCR that sample collection using a curette results in higher total bacterial counts compared to sampling with paper points. However, the proportions of periodontal bacteria examined (*A. actinomycetemcomitans*, *F. nucleatum*, *P. gingivalis*, *Pr. intermedia*, *Tr. denticola*, and *T. forsythensis*) were similar for both sampling techniques.
14. Samples can be comfortably stored at -80°C for extended periods with no measurable loss of antigen.
15. Immobilization of the antigen onto the solid support surface can be either a passive (non-specific) process such as adsorption or a more active chemical process, such as fixing proteins to the support surface irreversibly using glutaraldehyde. Polystyrene microtiter plates (Maxisorp Nunc, Denmark) are used in our laboratory as the support medium. Plaque suspended in the appropriate buffer has a high affinity for this surface although the manufacturers also provide a "certified" version of these plates to ensure maximal binding of the antigen to the surface.
16. Standard curves are prepared and run on every plate.
17. If the blood is not to be spun down straight away, keep vacutainer with blood at 4°C . If possible, separate serum from red cells in the same day.
18. The plate setup shown allows for eight serum samples at two concentrations to be assayed per plate (five wells are required for each sample, three wells for antibody concentration determination, and two for negative control wells). This configuration allows each individual serum sample to be corrected for its own background non-specific binding (15).
19. Boiling can also be performed in a thermocycler (100°C for 2 min) but use 0.2 mL or 0.5 mL PCR tubes.
20. Each rtPCR assay consisted of seven standards (positive controls) and a non-template (negative) control, in addition to the patient samples. Essentially, the relative concentration of bacterial DNA in each sample was determined by

comparing the amplification curve of each sample to those of the standards (16). Kirakodu et al. (17) have recently demonstrated that the use of actual cell counts to construct standard curves gave a lower estimate of bacterial numbers in mixed plaque samples compared with using a standard formula based on total DNA, although the log values were similar. These authors suggested that as long as the change is measured by logarithmic scale, either of the methods can be used.

21. Stock solutions containing all reagents except *Taq* polymerase can be prepared and stored up to 6 months at -80°C . Add *Taq* polymerase to the master mix immediately before use in PCR.
22. For simplicity and safety the agarose may be prepared in advance in a 250 mL Schott bottle. Any unused agarose can be allowed to solidify and reused (remelted).
23. Since EtBr is a known carcinogen, it can be replaced with SYBR[®] Safe DNA gel stain (Invitrogen, USA). SYBR[®] Safe is a non-hazardous alternative but it is heat sensitive and therefore must be added immediately before pouring the agarose gel.
24. The thickness of the agarose gel does not affect the resolution but only the time required to run the gel. At 110 V, a thin gel (20 mL of agarose) will run in ~ 30 min compared to thicker gel (100 mL) which could take up to 2 h.
25. The voltage used to run gels will influence the running time and resolution. When high resolution is required, use lower voltages (60 V) but if resolution is not a priority then higher voltages (120 V) can be used. Do not run gels above 120 V as the agarose will start to melt.
26. ABsoluteTM QPCR mix is a proprietary reaction buffer optimized for MgCl_2 concentrations to improve amplification across a wide range of template types.
27. Avoid touching the tops of the flat cap strips as the optics for the thermocycler sits directly above each reaction tube and fingerprints/smudges on the lids may affect the accurate detection of the fluorescent signal. Writing on the plates with markers should be avoided as some inks may also be fluorescent.
28. As “real-time” detection is used to display amplified DNA and no post-amplification modifications are needed, a 72°C extension is not required in the PCR cycle. If post-PCR modifications, such as sequencing or cloning, will be performed an extension cycle is advised.

29. When using TaqMan[®] probes, ensure that negative results (i.e., no fluorescent signal) are not the result of a single-nucleotide polymorphism (SNP) within the probe-binding region. Aliquots from the negative samples should be further examined by gel electrophoresis.

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References

1. Paster, B. J., Olsen, I., Aas, J. A., and Dewhirst, F. E. (2006) The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol* 2000. **42**, 80–87.
2. Socransky, S. S., and Haffajee, A. D. (2005) Periodontal microbial ecology. *Periodontol* 2000. **38**, 135–187.
3. Consensus report. (1996) Periodontal diseases: pathogenesis and microbial factors. *Ann. Periodontol.* **1**, 926–932.
4. Gmür, R., and Lüthi-Schaller, H. (2007) A combined immunofluorescence and fluorescent in situ hybridization assay for single cell analyses of dental plaque microorganisms. *J. Microbiol. Methods.* **69**, 402–405.
5. Woo, P. C. Y., Lau, S. K. P., Teng, J. L. L., Tse, H., and Yuen, K.-Y. (2008) Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin. Microbiol. Infect.* **14**, 908–934.
6. Socransky, S. S., Smith, C., Martin, L., Paster, B. J., Dewhirst, F. E., and Levin, A. E. (1994) “Checkerboard” DNA-DNA hybridization. *Biotechniques.* **17**, 788–792.
7. Hamlet, S. M., Taiyeb-Ali, T. B., Cullinan, M. P., Westerman, B., Palmer, J. E., and Seymour, G. J. (2007) *Tannerella forsythensis prtH* genotype and association with periodontal status. *J. Periodontol.* **78**, 344–350.
8. Jervøe-Storm, P. M., Alahdab, H., Koltzsch, M., Fimmers, R., and Jepsen, S. (2007) Comparison of curet and paper point sampling of subgingival bacteria as analyzed by real-time polymerase chain reaction. *J. Periodontol.* **78**, 909–917.
9. Kumar, P. S., Griffen, A. L., Moeschberger, M. L., and Leys, E. J. (2005) Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis. *J. Clin. Microbiol.* **43**, 944–955.
10. Bird, P. S., and Seymour, G. J. (1987) Production of monoclonal antibodies that recognize specific and cross-reactive antigens of *Fusobacterium nucleatum*. *Infect. Immun.* **55**, 771–777.
12. Hamlet, S. M., Cullinan, M. P., Westerman, B., Lindeman, M., Bird, P. S., Palmer, J., and Seymour, G. J. (2001) Distribution of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Prevotella intermedia* in an Australian population. *J. Clin. Periodontol.* **28**, 1163–1171.
12. Ratcliff, R. M., Chang, G., Kok, T., and Sloots, T. P. (2007) Molecular diagnostics of medical viruses. *Curr. Issues Mol. Biol.* **9**, 87–102.
13. Hamlet, S., Ellwood, R., Cullinan, M., Worthington, H., Palmer, J., Bird, P., Narayanan, D., Davies, R., and Seymour, G. (2004) Persistent colonization with *Tannerella forsythensis* and loss of attachment in adolescents. *J. Dent. Res.* **83**, 232–235.
14. Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Koikeguchi, S., Petelin, M., Arai, H.,

- Tanimoto, I., Nishimura, F., and Takashiba, S. (2003) Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *tetQ* gene and total bacteria. *FEMS Immunol. Med. Microbiol.* **39**, 81–86.
15. Yeung, S. C., Taylor, B. A., Sherson, W., Lazarus, R., Zhao, Z. Z., Bird, P. S., Hamlet, S. M., Bannon, M., Daly, C., and Seymour, G. J. (2002) IgG subclass specific antibody response to periodontopathic organisms in HIV-positive patients. *J. Periodontol.* **73**, 1444–1450.
16. Ford, P. J., Gemmell, E., Hamlet, S. M., Hasan, A., Walker, P. J., West, M. J., Cullinan, M. P., and Seymour, G. J. (2005) Cross-reactivity of GroEL antibodies with human heat shock protein 60 and quantification of pathogens in atherosclerosis. *Oral Microbiol. Immunol.* **20**, 296–302.
17. Kirakodu, S. S., Govindaswami, M., Novak, M. J., Ebersole, J. L., and Novak, K. F. (2008) Optimizing qPCR for the quantification of periodontal pathogens in a complex plaque biofilm. *Open Dent. J.* **2**, 49–55.

Chapter 10

Bacterial Viability Determination in a Dentinal Tubule Infection Model by Confocal Laser Scanning Microscopy

Abdul Aziz, Dikesh Parmar, Andrew McNaughton,
and Geoffrey R. Tompkins

Abstract

Dentinal tubule invasion protects bacteria from chemo-mechanical disinfection and frequently results in root canal treatment failures. *Enterococcus faecalis* is a primary causative agent, particularly in persistent, asymptomatic, and chronic apical periodontitis. In order to assess and compare the efficacies of endodontic antimicrobial agents and application strategies, we have developed a convenient and robust method to measure bacterial viability and assess distribution in an ex vivo tubule infection model. Following infection and antimicrobial treatment of prepared ex vivo roots, the tubule bacteria are exposed to nucleic acid-binding fluorescent stains (LIVE/DEAD® BacLight™ stain), sectioned, and examined by confocal laser scanning microscopy. The proportion of red-fluorescing (dead) and green-fluorescing (live) bacteria is then visualized in situ and quantified with image analysis software.

Key words: LIVE/DEAD® BacLight™ bacterial viability stain, confocal laser scanning microscopy, endodontic infection, *Enterococcus faecalis*, fluorescence.

1. Introduction

The primary concern in endodontic treatment is the elimination of viable bacteria within the root canal and adjacent dentinal tubules, to a degree that will favor a successful rehabilitation of the tooth. The development of effective antimicrobial agents and application techniques requires a satisfactory experimental endodontic infection system. The established model involves deliberate infection of sterilized ex vivo roots with known bacterial pathogens, such as *Enterococcus faecalis*, followed by

treatment with test medicaments and determination of the surviving bacteria (1). The difficulty with this approach is in determining the extent to which the antimicrobial treatments effectively penetrate the tubules, i.e., measuring the distance (from the canal) at which the medicament is no longer concentrated enough to kill the bacteria. Resilient organisms that invade and colonize the entire width of the tubules will, if not eradicated, cause subsequent re-infection of the restored tooth (2). Light microscopy and electron microscopy can be applied to visualize and measure bacterial penetration of the tubules in sectioned roots but not to distinguish between live and dead microorganisms (1, 3, 4). Enumeration of viable bacteria within the tubules has relied on conventional culturing techniques following progressive concentric reaming of the root from the canal outward (5). This approach is not only tedious to undertake but also inaccurate to the extent that most studies involving the culturing of tubule-invading bacteria avoid indication of the experimental variability.

The nucleic acid-binding fluorescent stains SYTO® 9 and propidium iodide (PI) have been applied in combination to differentiate live and dead bacteria by fluorescence microscopy (5). SYTO® 9 labels the nucleic acid of both live and dead bacteria because it penetrates intact biological membranes, but PI is unable to penetrate functional membranes and therefore only labels damaged bacteria (6). When both dyes interact with the same nucleic acid molecule, PI quenches the SYTO® 9 signal with the result that live bacteria fluoresce green and dead bacteria fluoresce red (7). These stains are available commercially as the LIVE/DEAD® *BacLight*™ bacterial viability stain and have been applied to environmental, food, and oral microbiological studies (8–10).

Weiger et al. (11) applied bacterial viability staining to measure microbial survival in the ex vivo human root dentine model following experimental infection and treatment. However, rather than visualize the bacteria in sectioned root specimens, these investigators progressively reamed the canal and examined the recovered shavings by fluorescence microscopy. Viability determinations were also made by conventional culturing of the shavings.

We have applied bacterial viability staining in conjunction with confocal laser scanning microscopy (CLSM) to quantify the effectiveness of intracanal antimicrobial agents in the experimental root infection model. This method requires relatively minimal specimen preparation and is considerably more accurate and more convenient than the bacterial culturing approach. Bacterial distribution and viability can be quantified both vertically and horizontally within the root, and multiple sections can be assessed within a single root.

2. Materials

2.1. Root Preparation

1. Sof-LexTM contouring and polishing Discs (ESPETM, 3 M, St. Paul, Minnesota, USA)
2. Diamond-coated crown burs (Dentsply Maillefer, Ballaigues, Switzerland)
3. 17% ethylenediaminetetraacetic acid cetrimide (EDTAC) (Dentalife Pty Ltd, Croydon, VIC, Australia)
4. 4% sodium hypochlorite (NaOCl) (Jasol, Auckland, New Zealand)
5. ISO size 10 Hedstrom files (Dentsply Maillefer, Ballaigues, Switzerland)
6. ProFile files (Dentsply Maillefer)

2.2. Root Infection

1. Brain-heart infusion medium (BHI; Becton Dickinson & Co., Sparks, MD, USA) supplemented with 0.5% (w/v) yeast extract (BHY)
2. Blood agar: BHI supplemented with 1.5% (w/v) bacteriological agar and 5% (v/v) defibrinated sheep blood
3. *E. faecalis* strain V583 (supplied by Prof. G. Cook, Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand) (*see Note 1*)

2.3. Root Staining

1. LIVE/DEAD[®] BacLightTM stain (Bacterial Viability Test, Invitrogen, Eugene, OR, USA)

2.4. Embedding and Sectioning

1. Disposable plastic spectrophotometric cuvettes (LP Italiana SPA, Milano, Italy)
2. VertexTM self-curing methyl methacrylate (Vertex-Dental B.V., Zeist, The Netherlands)
3. Precision cutting saw (Accutom-50, Struers, Ballerup, Denmark) fitted with 433CA blade (Struers) and CATSI specimen holder (Struers).

2.5. Confocal Laser Scanning Microscopy

1. Confocal laser scanning microscope (LSM 510 META NLO, Axiovert 200, Carl Zeiss Ltd., Jena, Germany)
2. ImageJ image processing software package (National Institutes of Health [NIH]; downloadable from <http://rsbweb.nih.gov/ij/index.html>)

3. Methods

3.1. Root Preparation

1. Intact single rooted premolars (extracted for either orthodontic or periodontal reasons) are preferred for the infection model. Immerse teeth in 5.25% sodium hypochlorite (NaOCl) (*see Note 2*) for 10 min in an ultrasonic instrument bath to remove organic deposits. Scrub all remaining persistent deposits with a stiff brush.
2. For teeth that have been stored in formalin, wash in running fresh tap water in an ultrasonic bath for 10 × 30-min cycles to remove residual formalin.
3. Decoronate the teeth with a water-cooled high-speed diamond bur.
4. Remove the cementum using an ESPETM disc attached to a slow-speed dental handpiece.
5. Immerse the prepared roots in 17% EDTAC for 5 min followed by 5 min in 5% NaOCl to remove the smear layer (*see Note 3*).
6. Rinse the roots thoroughly with either sterile water or sterile saline to remove NaOCl and EDTAC.
7. Confirm the patency of the roots using an ISO size 10 hand file. Set the working length by subtracting 0.5 mm from the length of the files when the tips are just visible at the apical foramen.
8. Prepare the roots to working length with rotary files using a crown-down technique.
9. Store roots at 4°C in sterile saline until required (*see Note 4*).

3.2. Root Infection

1. Cultivate *E. faecalis* on blood agar by anaerobic incubation at 37°C for 24 h. The agar culture can be stored at 4°C for 2–3 weeks but should be subcultured onto fresh medium after this period.
2. BHY is prepared in 20 mL volumes and autoclaved (121°C for 20 min). To some tubes, prepared roots are added and sterilized by autoclaving. Inoculate one BHY tube (without roots) with *E. faecalis* and incubate for 18–24 h.
3. Aliquots (200 µL) of the culture are added to each of the tubes containing sterilized roots and the tubes incubated at 37°C for 10 days. The growth medium is replenished every 48 h.
4. After the final replenishment of the medium, streak a sample onto BHI agar (incubate for 18 h) to confirm the purity of the culture.

5. Remove the roots from the culture medium using sterile forceps and at this stage antibacterial treatments are applied under appropriate conditions (i.e., relevant to clinical application).

3.3. LIVE/DEAD® BacLight™ Staining of the Root

LIVE/DEAD® BacLight™ stain (Bacterial Viability Test, Invitrogen, Eugene, OR, USA) was used in this method and any other similar stain may be used.

1. Following appropriate antimicrobial treatments, rinse the roots in sterile water and place in saline containing the LIVE/DEAD® BacLight™ stain preparation (using the distributor's recommended concentrations). At least one uninfected root should remain unstained for control and CLSM calibration.
2. Gently agitate the tubes manually for 30 s and place in the dark for 15 min.
3. Remove the roots using forceps and place in a 15 mL disposable plastic test tube (e.g., Falcon tube) containing sterile saline. Wash the roots thoroughly with three changes of saline and store at 4°C in saline (*see Note 5*).

3.4. Embedding and Sectioning the Root

1. For convenience, a maximum of nine 2 mL disposable plastic cuvettes can be joined together with cyanoacrylate to form a square (3 × 3) arrangement (*see Note 6*).
2. Position a minimal amount of PlayDoh (Hasbro Inc., Pawtucket, RI, USA) at the bottom of each cuvette and place one root in each cuvette with the coronal end embedded in the PlayDoh.
3. Press down to ensure that the root apices remain 1 mm below the rims of the cuvettes. This allows for accurate and uniform sectioning.
4. Place a colored marker in each cuvette for identification (*see Note 7*).
5. Make a very fluid mix of the powder and liquid of methyl methacrylate using double the recommended volume of the liquid and fill the cuvette. Place the blocks in a pressure flask at 2 bar for 45 min under cold water to minimize air bubbles. Inspect for bubbles after 45 min of pressure and, using a round bur carefully enlarge any bubbles, replenish with methyl methacrylate and re-pressurize. After a further 45 min, re-inspect the blocks and repeat procedure if necessary.
6. The block is now ready to section. The method below is specific to a Struers Accutom-50 (Struers, Ballerup, Denmark) but any precision cutting machine can be used for this purpose.

7. Attach the blade and tighten into place. Set the machine according to the type of blade.
8. Set the force at “medium,” blade speed at 1,200 rpm, and the feed speed at 0.05 mm/s. Program the machine for the required number of cuts, at 1 mm per cut.
9. Start the machine and ensure that a strong even coolant is spraying on the cutting edge of the blade. Adjust the jets to achieve this (*see Note 8*).
10. Attach the specimen holder to the cutting machine and mount the block of cuvettes on the holder. Move the holder in the *y*-axis to ensure that the block will be completely cut without touching the shaft of the blade. Set this as the “Stop” point.
11. Bring the block back to the edge of the blade and then adjust the *x*-axis until the blade is 1 mm from the edge of the cuvettes. Set this as the “Zero” point.
12. Make the first cut 1 mm from the edge of the cuvette block to exclude the 1 mm of uneven resin on the surface and to allow cutting machine to accurately determine the correct position of the block.
13. The machine will stop as soon as all the cuts have been made.
14. The resulting sectioned roots will be embedded within a frame formed by the nine bonded cuvettes (**Fig. 10.1**). Individual root sections can be separated from the matrix if required but this is not necessary for subsequent microscopy, and leaving sections in the 3 × 3 format may be more convenient.
15. Store all prepared sections refrigerated in saline (for up to 3 months) until ready for confocal laser scanning microscopy.

3.5. Confocal Scanning Laser Microscopy

An LSM 510 META NLO, Axiovert 200 (Carl Zeiss Ltd., Jena, Germany) was used in this method.

1. Turn the microscope on and open the software. The computer is then used to activate and adjust the parameters of the laser.
2. Configure the laser excitation lines by setting the filter at HFT 488/543. Arrange mirrors and plates depending on the channels chosen.
3. Select the argon laser and configure the band pass emission filter at 505–550 nm and the long pass filter at 650 nm (*see Note 9*).
4. Set the laser at 488 nm excitation and a power of 12%.

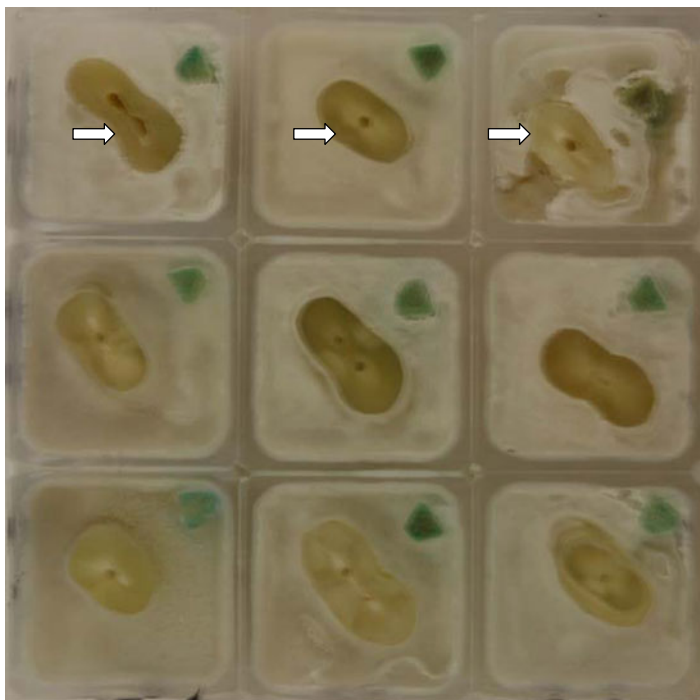


Fig. 10.1. Matrix of bonded cuvettes containing nine embedded roots after initial cross-sectional slice. *Arrows* indicate roots within methyl methacrylate block.

5. Adjust the objective lens to the desired magnification (*see Note 10*). Focus the image with halogen illumination and switch to the confocal mode using either the manual slide or the software (*see Note 11*).
6. Adjust the gain so that no fluorescence is detectable on an unstained, uninfected specimen (*see Note 12*).
7. Program the microscope software to generate an average from four passes of the optical slice. The data will be recorded and the distribution of voxels within the specimen calculated. Capture the resultant image and subsequently create a montage to cover the entire area of the sample (*see Note 13*).
8. Using the ImageJ image processing software (or equivalent), split the image obtained to show the green fluorescence (A), red fluorescence (B), and a composite image (C) (**Fig. 10.2**, *see Note 14*).
9. Prior to quantitative measurements, the minimum and maximum threshold parameters in ImageJ must be set to exclude the brightest and the dimmest voxels, thus eliminating any imaging artifacts.
10. Depth of penetration and percentage of green and red fluorescence can now be calculated using Image J.

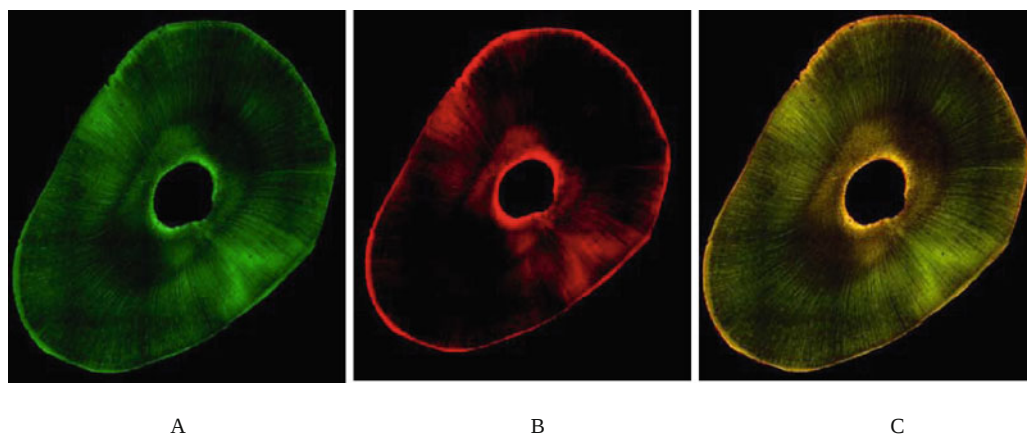


Fig. 10.2. Confocal laser scanning micrograph of a root cultured with *E. faecalis* for 10 days. **A** green fluorescence, **B** red fluorescence, and **C** composite image. The infected root has received antimicrobial treatment. The green fluorescence represents live bacteria and the red fluorescence shows dead bacteria. The composite image highlights regions (yellow/brown) containing both live and dead bacteria and regions of either predominantly live or dead bacteria.

4. Notes

1. Most ex vivo root infection models use *E. faecalis* but other species commonly associated with endodontic infection (e.g., *Pseudomonas aeruginosa*) may be used with minor changes (such as the culture medium and incubation conditions).
2. It is important to use a high concentration of NaOCl to thoroughly dissolve any remnants of organic tissue.
3. When using EDTA, other formulations such as EDTAC and REDTAC can also be used to remove the smear layer. Removing the cementum allows dentinal tubules to open on both ends allowing easier penetration of bacteria.
4. Ensure that the roots are never allowed to dry out as the dentine will crack. Store the roots refrigerated at all times to avoid fungal growth.
5. Immediately following staining, wash the excess stain from the roots, otherwise the PI will label bacteria as they die subsequent to the experiment giving misleading results.
6. Using the cuvettes allows a uniform container for housing the roots. When joining the cuvettes, line up the edges so that they are flush with each other. A slight discrepancy will result in significantly uneven dimensions on cutting. Mark a

‘V’ (or other tapering shape) on each side of the block with indelible marker. This facilitates convenient identification and ordering of consecutive slices. When placing the roots in the cuvette, ensure that the apical ends are 1 mm below the mouth of the cuvette. Make a jig out of silicon putty by taking an impression of the cuvette opening and trimming the impression to allow 1 mm of the impression material from the cuvette edge. Use this jig to push the roots into the PlayDoh.

7. Color-coded burnout dental post can be used as the markers embedded within the cuvettes.
8. The coolant spray for the Accutom-50 must remain constant on the cutting edge or the blade will break causing damage to the machine and the specimen. The metal filters must be cleaned regularly for the coolant to flow through.
9. Two different filters are used because SYTO[®] 9 has a lower excitation peak value than PI.
10. When changing objectives on the confocal microscope, be aware of what specimen you have on the stage, particularly if an unusually thick specimen is used, as damage to the objectives and the specimen is possible during rotation of the nosepiece.
11. CLSM requires the sample to be in focus for a true fluorescent image. An image of the root section must only be considered valid when the tubules are visible. If the color is visible but the tubules are not obvious or the indentations caused by the saw are visible, the image must be refocused. When storing the images, record as much detail as possible to identify the specimen later as a large volume of data may be collected.
12. Setting the confocal microscope such that in the absence of a sample no fluorescence is visible ensures that the images obtained are of the stained samples and not background noise or auto-fluorescence.
13. To create a montage, the x - and y -axes have to be set on the microscope stage with the number of individual images required. Do this by positioning the laser in the center of the sample. Choose the number of images required in the x - and y -axes according to the length and width of the specimen.
14. Observe all images in a room with no ambient light. This ensures much brighter and sharper fluorescent images. Superimposing the green and red will highlight areas with mixed live and dead bacteria as well as areas with a predominance of either live or dead cells.

References

1. Haapasalo, M., and Ørstavik, D. (1987) In vitro infection and disinfection of dentinal tubules. *J. Dent. Res.* **66**, 1375–1379.
2. Engstrom, B. (1964) The significance of *Enterococci* in root canal treatment. *Odontol. Revy.* **15**, 87–106.
3. Ørstavik, D., and Haapasalo, M. (1990) Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. *Endod. Dent. Traumatol.* **6**, 142–149.
4. Love, R. M. (2002) The effect of tissue molecules on bacterial invasion of dentine. *Oral Microbiol. Immunol.* **17**, 32–37.
5. Virta, M., Lineri, S., Kankaanpää, P., Karp, M., Peltonen, K., Nuutila, J., and Lilius, E. M. (1998) Determination of complement-mediated killing of bacteria by viability staining and bioluminescence. *Appl. Environ. Microbiol.* **64**, 515–519.
6. Mason, D. J., Allman, R., Stark, J. M., and Lloyd, D. (1994) Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. *J. Microsc.* **176**, 8–16.
7. Auty, M. A., Twomey, M., Guinee, T. P., and Mulvihill, D. M. (2001) Development and application of confocal scanning laser microscopy methods for studying the distribution of fat and protein in selected dairy products. *J. Dairy Res.* **68**, 417–427.
8. Boulos, L., Prevost, M., Barbeau, B., Coallier, J., and Desjardins, R. (1999) LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Methods* **37**, 77–86.
9. Gao, M. S., Wilks, S. A., Azevedo, N. F., Vieira, M. J., and Keevil, C. W. (2009) Validation of SYTO 9/propidium iodide uptake for rapid detection of viable but noncultivable *Legionella pneumophila*. *Microb. Ecol.* **58**, 56–62.
10. Leuko, S., Legat, A., Fendrihan, S., and Stan-Lotter, H. (2004) Evaluation of the LIVE/DEAD BacLight kit for detection of extremophilic archaea and visualization of microorganisms in environmental hypersaline samples. *Appl. Environ. Microbiol.* **70**, 6884–6886.
11. Weiger, R., de Lucena, J., Decker, H. E., and Lost, C. (2002) Vitality status of microorganisms in infected human root dentine. *Int. Endod. J.* **35**, 7166–7171.

Chapter 11

Characterization of Anti-competitor Activities Produced by Oral Bacteria

Fengxia Qi and Jens Kreth

Abstract

Most bacteria in nature exist in multispecies communities known as biofilms. In the natural habitat where resources (nutrient, space, etc.) are usually limited, individual species must compete or collaborate with other neighboring species in order to perpetuate in the multispecies community. The human oral cavity is colonized by >700 microbial species known as the indigenous microflora. This indigenous flora normally maintains an ecological balance through antagonistic as well as mutualistic interspecies interactions. However, environmental perturbation may disrupt this balance, leading to overgrowth of pathogenic species, which could in turn initiate diseases such as dental caries (tooth decay) and periodontitis (gum disease). Understanding the mechanisms of diversity maintenance may help development of novel approaches to manage these “polymicrobial diseases.” In this chapter, we will focus on a well-characterized form of biochemical warfare: bacteriocins produced by *Streptococcus mutans*, a primary dental caries pathogen, and H₂O₂ produced by *Streptococcus sanguinis*, an oral commensal. We will describe detailed methodologies on the competition assay, isolation, purification, and characterization of bacteriocins.

Key words: Bacteriocins, oral streptococci, interspecies competition, biofilms, luciferase reporter.

1. Introduction

Most of the antibiotics we use today are produced by microbes, and it is estimated that >99% of bacterial species in nature produce some type of antibiotics (1–3). Although the ecological role of these antibiotics is less studied, it is clear that their production is for the protection of the producing species against other microbes (2). Bacteriocins are peptide antibiotics. Unlike the traditional antibiotics, which are produced as secondary metabolites, bacteriocins are synthesized ribosomally. In general, there are two types

of bacteriocins, the lantibiotics and the non-lantibiotics. The lantibiotics are extensively modified peptides, containing dehydrated threonine and serine residues and thioether bridges (4), while the non-lantibiotics are unmodified peptides, which can comprise one or two components for activity. Bacteriocin production appears to be prevalent; nearly all sequenced bacterial genomes encode bacteriocin-like genes, although most of them have not been characterized.

Inter-species interactions among microbial species within the same communities are well-documented phenomena in scientific literature. The dental biofilm is a good model system for studying interspecies interactions owing to its vast biodiversity (>700 bacterial species) (5–8), high cell density (10^{11} cells/g wet weight) (9), and easy accessibility. In addition, the oral cavity is an environment with constant cycles of feast and famine and fluctuations of pH due to food intake from the host. The high density and diversity of oral biofilm community members coupled with a limited food supply creates an environment that is conducive to fierce competition for available resources.

Streptococcus mutans is considered a major pathogen causing human dental caries (also known as tooth decay) (10). *S. mutans* is a copious producer of both types of bacteriocins (named mutacins). *Streptococcus sanguinis* is an oral commensal residing in the same oral biofilm community as *S. mutans*. Except for reported associations with bacterial endocarditis, *S. sanguinis* is considered a benign, or even a beneficial, bacterium with regard to dental caries (11, 12). The antagonism between *S. mutans* and *S. sanguinis* at the ecological level has been known for many years (12, 13). Our group started investigating the mechanisms of interspecies interaction between *S. mutans* and *S. sanguinis* over 10 years ago. We have shown that mutacin production by *S. mutans* and H_2O_2 production by *S. sanguinis* play an important role in the competition between the two species (14, 15). Techniques described in this chapter were developed from these studies; however, they can be easily adapted to studying interspecies interactions among other species.

2. Materials

2.1. Bacteriocin Assay

1. BHI or TH agar plates: dissolve 37 g/L Brain-Heart Infusion (BHI) or 30 g/L Todd-Hewitt broth (TH) in deionized water (DI H_2O), add 15 g bacteriological agar. Autoclave at 121°C for 30 min. Let cool to ~55°C and pour plates. Half-strength BHI or TH contains 18.5 g/L (BHI) or 15 g/L (TH), respectively (*see Note 1*).

2. BHI or TH soft agar: same as above, but use 7.5 g agar. After autoclaving, dispense 4 mL aliquots into glass tubes and store at 4°C. Before use, melt the agar in boiling water, or in a microwave inside a water-filled beaker.

2.2. Biofilm Assay and Confocal Laser Scanning Microscopy

1. Lab-Tek II Chamber Slide™ System (Nalge Nunc International; Naperville, IL, USA).
2. CellTracker™ Orange CMTMR (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) (Molecular Probes; Eugene, OR, USA), store at -70°C.
3. Sucrose (20% stock) in DI H₂O, filter-sterilized (do not autoclave).
4. Confocal laser scanning microscope.

2.3. H₂O₂ Assay

1. 10 mM phosphate buffer (pH 7.4), make 100 mM stock solution by mixing 19 mL of 100 mM monobasic sodium phosphate and 81 mL of 100 mM dibasic sodium phosphate. Filter sterilize and store at room temperature.
2. *o*-Dianisidine dihydrochloride (ICN, Aurora, OH).
3. Horseradish peroxidase (Pierce, Rockford, IL).
4. Triton X-100 (Sigma).
5. Leuco crystal violet (Sigma), dissolve powder directly into BHI agar medium (after autoclaving), and pour plates.
6. 30% H₂O₂ (Sigma, St. Louis, MO).
7. CO₂ incubator for aerobic incubation.

2.4. Isolation and Purification of Bacteriocin

1. Pharmacia AKTA Purifier (GMI).
2. Trifluoroacetic Acid (TFA), (Thermo Scientific), make a 0.1% solution with HPLC-grade DI H₂O, store at room temperature.
3. Methanol (HPLC grade) (Cole-Parmer), make 85% solution with HPLC grade DI H₂O. Store at room temperature.
4. Acetonitrile – HPLC grade (Fisher Scientific).
5. Chloroform (Sigma).
6. Urea (Fluka), make 5 M with DI H₂O, store at room temperature.

2.5. Derivatization of Lantibiotics

1. 100% ethanol (Sigma)
2. 5 M NaOH in DI H₂O, store at room temperature
3. Ethanethiol (Sigma)
4. Acetic acid – glacial (Sigma)

2.6. Cloning and Other Genetic Techniques

1. Restriction enzymes (New England Biolabs, Ipswich, MA, USA), store at -20°C .
2. DNA ligase (New England Biolabs, Ipswich, MA, USA), store at -20°C .
3. Elongase enzyme mix (a mixture of *Pyrococcus* sp. thermostable DNA polymerase and *Taq* DNA polymerase) (Invitrogen). Store at -20°C .
4. TOPO[®] TA Cloning[®] kit (Invitrogen). Store at -20°C .
5. *Escherichia coli* DH5 α competent cells (Invitrogen). Store at -70°C .
6. Ampicillin (Fluka), 100 mg/mL stock dissolved in 50% ethanol, store at -20°C , use at 100 $\mu\text{g/mL}$ final concentration.
7. Kanamycin (EMD), 100 mg/mL stock dissolved in DI H_2O , store at -20°C , use at 100 $\mu\text{g/mL}$ final concentration.
8. Spectinomycin (Sigma), 150 mg/mL stock in DI H_2O , store at -20°C , use 150 $\mu\text{g/mL}$ final concentration.
9. LB broth (DIFCO).
10. Agar (DIFCO).

3. Methods

3.1. Competition Assay on Plate Culture

1. Since most, if not all, bacteriocins are produced under high cell density, plate cultures are usually used to analyze interspecies competition. Here we use an example of competition between *S. mutans* and *S. sanguinis*. The assay can be done by inoculating either species first as the “early” colonizer, then inoculating the other species after overnight growth as the “late” colonizer. Additionally, one could inoculate both species at the same time, i.e., a simultaneous antagonism experiment.
2. Usually, an overnight culture is adjusted to an optical density at 600 nm (OD_{600}) of 0.5 in 50% BHI and 10 μL is spotted onto half-strength (50%) BHI plates as the early colonizer.
3. After an overnight incubation, 10 μL of the competing species, also adjusted to the same OD_{600} , is spotted beside the early colonizer as the late colonizer, or both species are inoculated at the same time beside each other (simultaneous antagonism). The plates are further incubated at 37°C

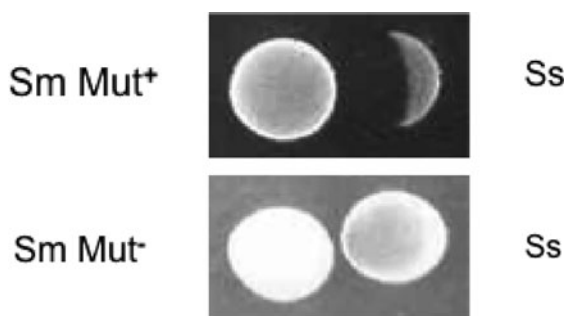


Fig. 11.1. Interspecies competition assay between *Streptococcus mutans* (Sm) and *Streptococcus sanguinis* (Ss). Mut⁺ = wild-type mutacin producer; Mut⁻ = mutacin mutant.

anaerobically overnight before cell growth is inspected. A typical outcome between a pair of true competitors is illustrated in **Fig. 11.1**. In this example, when the bacteriocin gene from *S. mutans* is inactivated (Mut⁻), *S. sanguinis* is no longer inhibited.

3.2. Competition Assay in Biofilms

1. For competition assays in biofilms, overnight cultures of *S. mutans* or *S. sanguinis* are diluted 1:100 in 50% BHI plus 0.1% sucrose and inoculated into a Lab-Tek II Chamber SlideTM.
2. The cultures are incubated at room temperature for 3 h to allow cell attachment before the competing species is inoculated, or both species are inoculated at the same time.
3. The biofilm is grown for 16 h at 37°C as a static culture. CellTracker Orange is used to label all cells for 2 h before confocal microscopy.
4. For microscopy the Lab-Tek II Chamber SlideTM System is modified: the objective slide is replaced by a thin cover-slide for proper CLSM microscopy since most microscope lenses have a shorter working distance and image acquisition would be obscured by the thick objective slide.
5. CLSM is performed with a microscope equipped with detectors and filter sets for monitoring red fluorescence (excitation wave-length 540–580 nm [560 CWL], dichroic mirror wavelength: 595 nm [LP], barrier wavelength 600–660 nm [630 CWL]). Images might be obtained with a 10 × 0.3 Plan-Neofluar and a 40 × 1.4 Plan-Neofluar oil objective.

3.3. H₂O₂ Production Assay

1. H₂O₂ production by *S. sanguinis* plays an important role in interspecies competition with *S. mutans* (14). The production of H₂O₂ by *S. sanguinis* in aerated liquid culture is measured as follows: samples (1 mL) are taken at the desired time

points, centrifuged (16,000*g*) for 5 min, and transferred (0.2 mL) to a new incubation tube.

2. A reaction solution is prepared fresh for each experiment (0.8 mL of 10 mM phosphate buffer [pH 7.4] with 0.16 mM *o*-dianisidine dihydrochloride [ICN, Aurora, OH], 1.2 μ g/mL of horseradish peroxidase [Pierce, Rockford, IL], 0.02% Triton X-100) and added to the reaction mixture followed by incubation at 37°C for 20 min.
3. The absorbance at 570 nm is determined, and the concentration is calculated from a standard curve prepared for each experiment from a 30% H₂O₂ stock solution ranging from 0 to 500 nmole/mL (Sigma, St. Louis, MO).
4. To measure the effect of *S. mutans* on the H₂O₂ production of *S. sanguinis*, an overnight culture of *S. sanguinis* is diluted to 10⁷ cells/mL (OD₆₀₀ = 0.025) and incubated aerobically at 37°C. After two doubling times, the cells are washed twice with BHI and the OD₆₀₀ is adjusted to 0.2.
5. One milliliter of the cell suspension is transferred to a tube, and 1 mL of either BHI or *S. mutans* cell suspension (OD₆₀₀ = 0.2) is added. The cells are further incubated either as a planktonic culture or as a cell pellet with medium (16,000*g* for 1.5 min) for 2 h before the H₂O₂ concentration is measured with the culture supernatant.
6. For the determination of H₂O₂ production on the plate, 10 μ L of peroxidase (64 μ g) is added to a half-strength BHI plate containing 1 mg/mL leuco crystal violet. After the liquid is absorbed into the agar, 5 μ L of *S. sanguinis* is inoculated at the same spot. After overnight incubation in a CO₂ incubator, the plate is inspected for the development of a purple color on and around the colony.

3.4. Bacteriocin Activity Assay by Deferred Antagonism (Plate Overlay)

1. To isolate bacteriocin producing bacteria from saliva, unstimulated whole saliva is collected by asking the volunteers to expectorate into a sterile 1.5-mL microcentrifuge tube.
2. The saliva is first diluted 1:10 in phosphate-buffered saline (PBS), and cells are dispersed by vortexing for 1 min. A 10-fold serial dilution is performed with the cell suspension, and a portion (100 μ L) of each dilution is plated on BHI or TH plates.
3. The plates are incubated for 2 days in an anaerobic chamber with 90% N₂, 5% CO₂, and 5% H₂ at 37°C, or in a candle jar in a regular 37°C incubator. Plates with well-separated single colonies can be overlaid directly with an indicator strain, or colonies can be transferred to a new plate with toothpicks

and grown for 1–2 days until colonies are formed, and then the plate overlaid with an indicator strain.

4. The indicator strain is grown in BHI or TH broth overnight, and 0.5 mL of the overnight culture is mixed with 4 mL of melted BHI or TH soft agar cooled to $\sim 50^{\circ}\text{C}$. The mixture is then poured onto the plate and incubated overnight under the same conditions. A zone of inhibition of the indicator strain suggests production of a bacteriocin (**Fig. 11.2**).

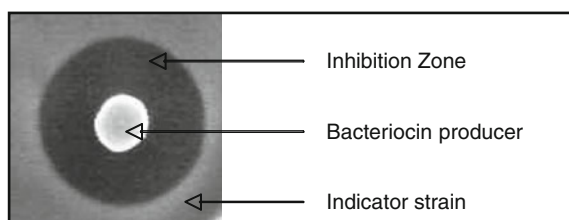


Fig. 11.2. Bacteriocin production assay.

3.5. Isolation of Bacteriocin

1. Since most bacteriocins are produced when cell density is high, plate culture is used initially to isolate bacteriocin (*see Note 2*). In the case of mutacin I ([16](#)), TH plates are made, which contain 0.3% agarose in place of agar. A sterile PHWP membrane (0.5 μm pore size, Millipore) is placed on the plate surface, and an overnight culture of the producing strain is spread onto the membrane. The plate is incubated for 2 days for the bacterial lawn to form on the membrane, and the membrane is then transferred onto a new plate. This process is repeated up to 8 transfers or until the bacterial lawn stops to produce bacteriocin. This should be tested with the overlay assay on a separate plate.
2. The spent plate is frozen at -70°C and thawed quickly at 60°C in a water bath. Upon freezing-and-thawing, the agarose would disintegrate to release the liquid content containing the bacteriocin. The liquid phase is separated from the agarose debris by centrifugation (20,000g for 30 min).
3. Mutacin is extracted from the liquid phase by equal volumes of chloroform. The emulsion at the chloroform–aqueous interface, which contains mutacin I, is collected by centrifugation, and the pellet dried under a stream of air.
4. The pellet is suspended in 5 M urea. To assay for activity of the crude mutacin extract, a 2-fold dilution of the crude extract is made and 10 μL of each dilution is spotted onto a pre-dried TH plate. After the liquid spot is dry, the plate is overlaid with the indicator strain (*see Section 3.1*), and the plate is incubated overnight at 37°C anaerobically. One arbitrary unit of activity is defined as the highest dilution

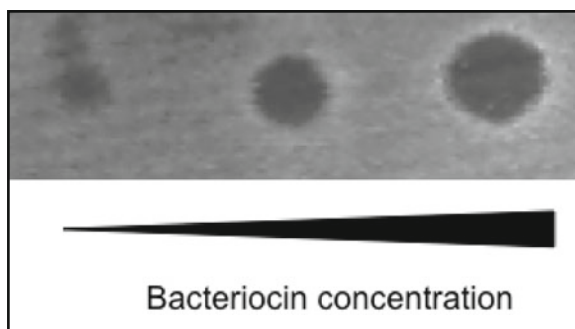


Fig. 11.3. Bacteriocin titer determination.

that exhibits a clear zone of inhibition of the indicator strain (**Fig. 11.3**).

3.6. Purification of Bacteriocin

1. Since all bacteriocins are small peptides, reverse phase HPLC is generally used for purification. In the case of mutacin I and III (**17**), the crude extract is applied to a Source 15RPC column and eluted with a fragmented gradient of buffer A (0.1% TFA) and buffer B (0.085% TFA in 80% methanol) with the AKTA purifier and the UNICORN control system (Amersham Pharmacia Biotech, Piscataway, N.J.) (*see Note 3*).
2. A 1-mL eluent is collected and tested for activities using the methods described in **Section 3.2**.
3. The active fractions are pooled and dried in a lyophilizer. The pellet is re-dissolved in 0.25% TFA and subjected to a second round of purification with the same column and protocol.
4. The single active peak fraction is collected, dried in a lyophilizer, and used for sequence analysis and electrospray-ionization mass spectrometry (EIMS). A typical HPLC profile is presented in **Fig. 11.4**.

3.7. Sequencing of the Purified Bacteriocin

1. For non-modified bacteriocins, a simple *N*-terminal peptide sequencing can be performed using automated Edman degradation by any protein sequencing service.
2. For lantibiotics, chemical modifications of the peptide should be made to reduce the thioether bridges and dehydrated amino acids prior to sequencing via automated Edman degradation procedures (*see Note 4*).
3. For chemical modification, 50 μg of purified mutacin I is dried under vacuum and resuspended in 90 μL of a derivatization mixture consisting of 280 μL of ethanol, 200 μL of water, 65 μL of 5 M sodium hydroxide, and 60 μL of ethanethiol. The reaction proceeds at 50°C for 1 h under

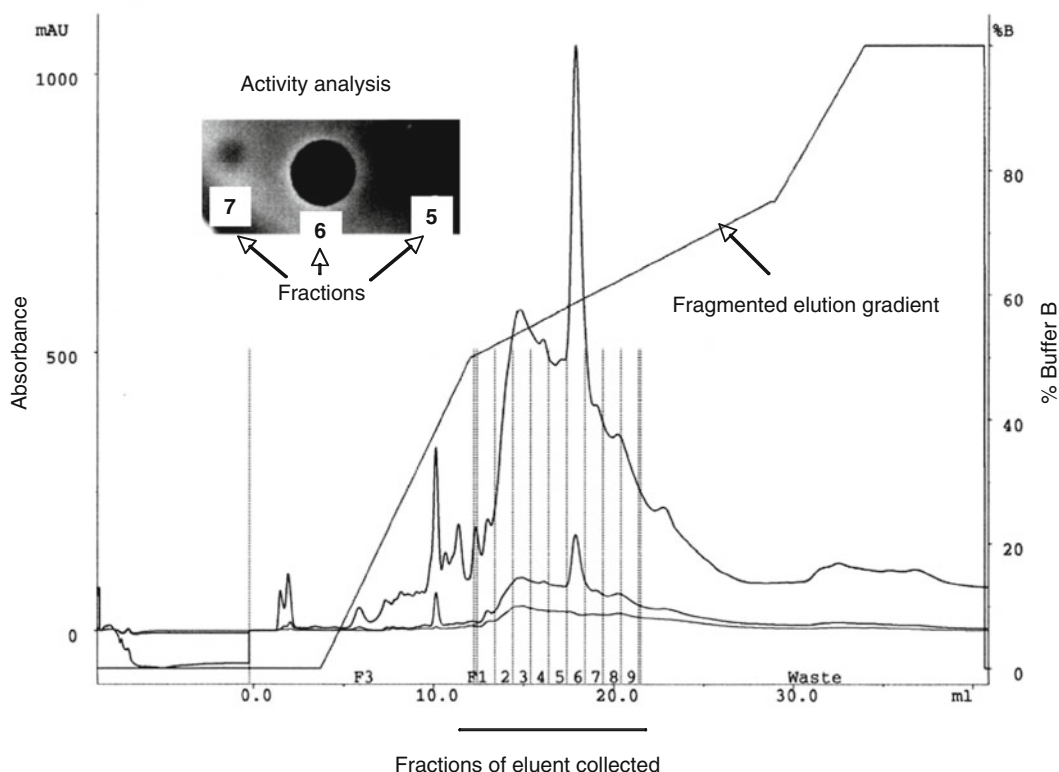


Fig. 11.4. HPLC profile of mutacin I.

nitrogen and is then stopped by the addition of 2 μL of acetic acid. The reaction mixture is dried under vacuum and washed three times with 50% ethanol. The pellet is resuspended in 10 μL of 50% acetonitrile with 1% formic acid for EIMS analysis and *N*-terminal peptide sequencing by Edman degradation.

3.8. Isolation of Bacteriocin Structural Genes by Reverse Genetics

1. After sequencing the bacteriocin peptide, the structural gene can be isolated via a circular PCR strategy (*see* Fig. 11.5). Generally, a pair of degenerate primers is designed based on the peptide sequence and the codon usage of the producing strain. One primer (reverse) is pointing upstream from the 5' portion of the derived DNA fragment and the other (forward) faces downstream from the 3' portion of the DNA fragment.
2. The chromosomal DNA of the producing strain is digested to completion with a panel of restriction enzymes and self-ligated. The ligation mixtures are used as templates in PCR reactions with the reverse and forward primers (*see* Notes 5, 6 and 7).

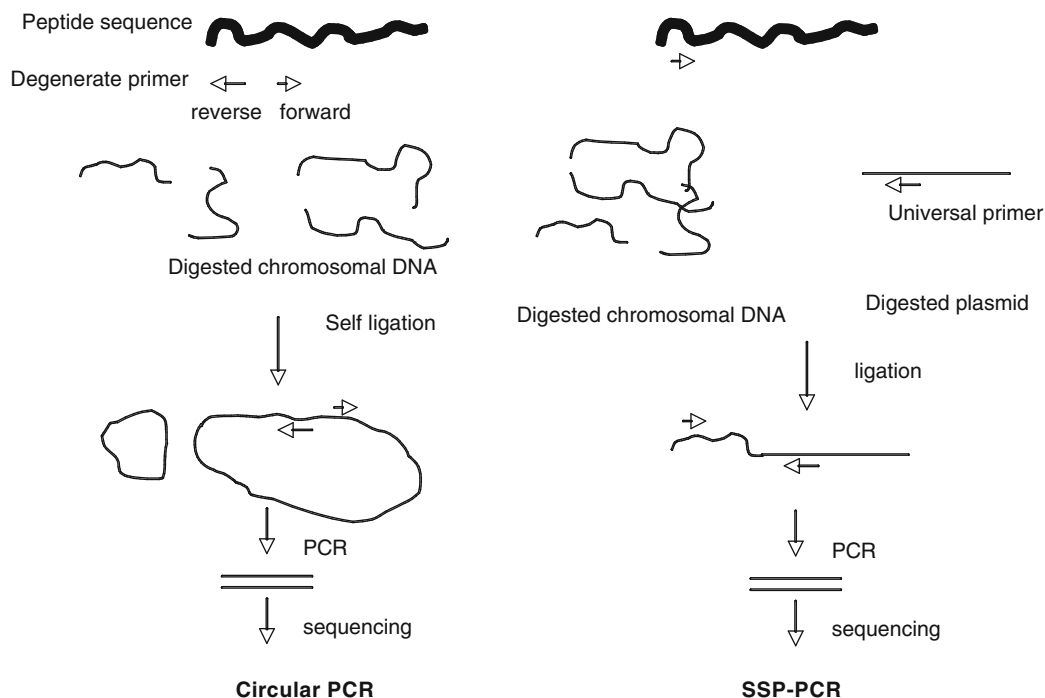


Fig. 11.5. Strategies for cloning a bacteriocin gene via reverse genetics.

3. The PCR products are then cloned and sequenced. The upstream and downstream sequences could be distinguished at the unique restriction site where the chromosomal DNA is initially cut. With the upstream and downstream sequences available, the structural gene can be re-confirmed by regular PCR using primers designed based on the upstream and downstream sequences.
4. Alternatively, the structural gene can be obtained by a single specific primer PCR (SSP-PCR) (see Fig. 11.5). In this strategy, a degenerate primer is designed based on the peptide sequence.
5. The chromosomal DNA is digested by a set of restriction enzymes to completion, and the same set of enzymes are used to digest a commonly-available cloning vector such as pUC or pBluescript vectors.
6. The same enzyme digested chromosomal DNA and the plasmid is ligated, and the ligation mixture is used as template for PCR with the specific primer and one of the universal primers.
7. The PCR product is then sequenced using the universal primer.

8. For both strategies, 1 mg of chromosomal DNA is normally used for digestion in 20 μ L reaction mixture. The PCR conditions are 94°C for 4 min, 50°C for 1 min, and 72°C for 5 min for 1 cycle; 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min for 25 cycles; a final cycle at 94°C for 1 min, 50°C/1 min, and 72°C/10 min. The PCR reaction contains a mixture of *Taq* and *Pfu* DNA polymerases and to ensure high processivity and fidelity.

3.9. Mutagenesis via Single and Double Crossover

1. To study the function of the bacteriocin in interspecies competition, an isogenic strain defective in bacteriocin production is needed. Two strategies can be used to inactivate the bacteriocin biosynthesis gene by homologous recombination: (a) single crossover insertional inactivation, and (b) allelic replacement via a double crossover mechanism (**Fig. 11.6**).
2. Single crossover is utilized for mutagenesis of a gene, which is either in a single gene system or is the last gene in a multigene operon. In general, a $\sim$ 300 bp internal fragment of the target gene is amplified by PCR using primers with restriction sites incorporated at the 5' ends. The PCR product is then digested with the appropriate restriction enzyme and ligated into a suicide vector (i.e., pFW5) digested with the same enzyme. The ligation mixture is transformed into *E. coli*, and the recombinant plasmid is isolated from positive clones.
3. The plasmid is then transformed into the bacteriocin-producing strain via natural transformation or electroporation depending on the specific strain. Transformants are propagated on selective agar plates and tested for bacteriocin production by using the plate overlay method (*see Note 8*).
4. A double crossover strategy is used to specifically inactivate, usually by insertion of an antibiotic cassette, individual or multiple genes in an operon in order to avoid polar effects on the downstream gene. The antibiotic cassette typically contains its own promoter but lacks a transcription terminator. For double crossover, the simplest method is 3-piece PCR ligation. Briefly, a 1-kb fragment of the upstream and downstream regions of the target gene, as well as the antibiotic cassette, is amplified by PCR. In the primers at the junction of each fragment, an 18-nucleotide overlapping sequence is incorporated in the primer, which is homologous to the antibiotic cassette sequence (*see Note 9*).
5. After the first PCR, the three fragments are purified with a commercial PCR purification kit (e.g., QIAGEN QIAquick kit) and eluted with 40 μ L elution buffer. One microliter of each fragment is then mixed and used as template for a

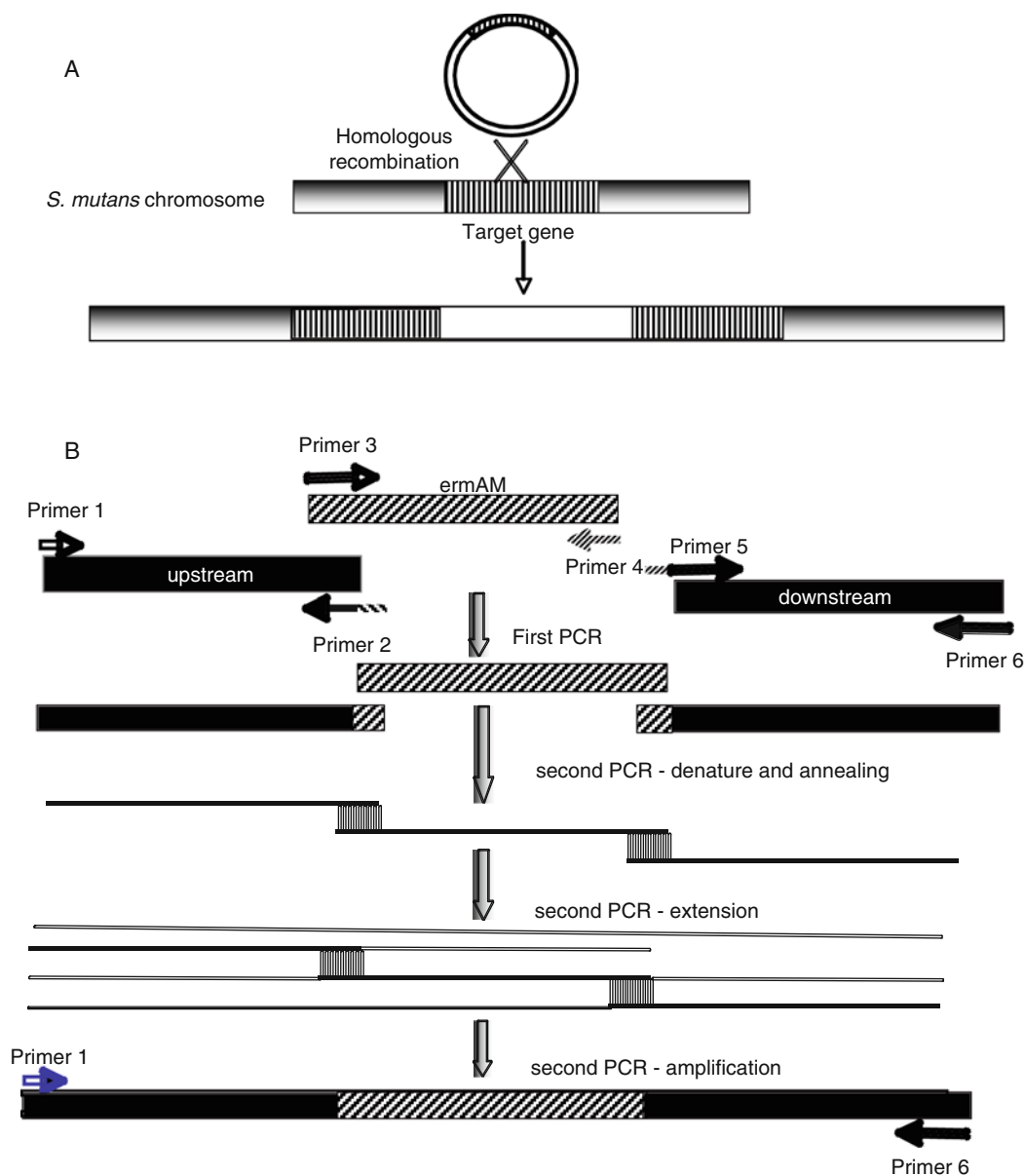


Fig. 11.6. **A.** Insertional inactivation by single-crossover integration. **B.** Construction of an allelic replacement construct via three-piece PCR ligation strategy.

second PCR employing the two primers on both ends of the ligated fragment. The PCR product is purified using a spin column and transformed directly into the wild-type bacteriocin producer strain. Transformants are selected on selective plates and subsequently tested for bacteriocin production.

3.10. Gene Expression Analysis by Reporter Fusions

1. The firefly luciferase is a good reporter for quantification of promoter activities. For constructing a reporter gene fusion, the promoter region of the target gene is amplified from

chromosomal DNA with primers incorporating restriction enzyme sites and cloned into plasmid pFW5-luc (18). pFW5 is a suicide plasmid, which replicates in *E. coli* but not in *S. mutans* unless it is integrated into the chromosome via homologous recombination at the promoter locus.

2. After integration into the chromosome, the promoter activity of the target gene under different conditions can be monitored by measuring the luciferase activity. The luciferase activity should be normalized to cell density or protein content of the sample.

3.11. Luciferase Assay Using Live Cells

1. To test for luciferase activity, 25 μL of 1 mM D-luciferin (Sigma; St Louis, MO) suspended in 100 mM citrate buffer, pH 6, is added to 100 μL of the cell culture.
2. To ensure sufficient levels of intracellular ATP pools for luciferase activity, cells are recharged with 1% glucose for 10 min prior to luciferin addition.
3. Luciferase activity is measured by using a TD 20/20 luminometer (Turner Biosystems; Sunnyvale, CA).

4. Notes

1. Bacteriocin production is sensitive to the growth conditions and the detection requires a sensitive indicator strain. The absence of a zone of inhibition when tested under a particular condition does not necessarily mean the strain is a non-producer. One needs to test the producer bacterium under different conditions and employing different indicator strains. Our experience suggests that bacteriocin production is a stress response. Therefore, growth of the strain in a very rich medium such as BHI tends to inhibit bacteriocin production. Hence, diluted BHI or TH could be used. Interestingly, a very nutrient-poor medium is also not conducive to bacteriocin production. Another important observation is that bacteriocin production is more prevalent among newly-isolated clinical strains, and the ability tends to diminish or disappear upon repeated laboratory passage.
2. Although some bacteriocins can be produced by planktonic (broth) culture grown to late logarithmic or early stationary phase, the production level is usually lower than that observed on solid media. For initial isolation, it is important that the yield is high. Another advantage of using a culture grown on an agar plate is that an overlay can be made on a parallel plate culture to verify that the bacteriocin is indeed

produced. Using the filter membrane as a supporting substratum for the bacterial lawn favors bacteriocin production. In our experience, the highest yield is obtained at passages 2–5, and the yield declines after passage 6, possibly due to the aging of the bacterial population. Another advantage of using a membrane is that no subsequently filtering of the supernatant is necessary, because there is no bacterial cell contamination of the agarose plate. The membrane allows bacteriocins to diffuse into the medium underneath while preventing bacterial cells from going through.

3. As mutacins I and III are fairly hydrophobic molecules, chloroform is used for their extraction. For bacteriocins that are less hydrophobic, ammonium sulfate ($[\text{NH}_3]_2\text{SO}_4$) precipitation can be used. For HPLC-based purification, a linear gradient is typically used initially to determine at what fraction the active component is eluted, then a fragmented elution is used to further separate other components from the active one if the active peak does not appear to be pure (a pure peak is usually smooth and symmetrical in shape).
4. N-terminal peptide sequencing using automated Edman degradation chemistry is blocked by dehydrated amino acids or thioether bridges. If this happens during sequencing of the purified bacteriocin, it would suggest that the bacteriocin is a lantibiotic. The number of dehydrated amino acids and thioether bridges can be deduced by comparing the molecular mass of the peptide and its modified form by EIMS.
5. The circular PCR or SSP-PCR strategy is used because it is normally difficult to obtain a full-length sequence of the bacteriocin peptide, and this is especially true for lantibiotic peptides. However, a 6–7 amino acid sequence from the N-terminus is relatively easy to obtain. Therefore, by using these strategies, forward and reverse degenerate primers can be designed based on this short sequence in order to “fish out” the structural gene.
6. An important factor to consider using circular PCR is the concentration of the digested chromosomal DNA for self-ligation. To facilitate self-ligation (intramolecular ligations), less DNA is better. Our experience is to set up a series of ligations with different concentrations of DNA and use 1 μL of each concentration in PCR experiments.
7. Available sequenced genomes can be used to screen for bacteriocin genes, if the producer bacterium is known (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>).
8. One caveat for the single crossover strategy is that if the gene is too small, like in the case of most bacteriocin structural genes, it will be difficult to inactivate by the single crossover. In this case, an allelic replacement should be used.

9. One caveat for the double crossover strategy is that the expression pattern of the downstream genes may be changed because all of them will be transcribed from the antibiotic cassette promoter. This could create a problem for genes whose expression pattern is important for its biological function. One strategy to overcome this problem is to include a transcription terminator at the 3' end of the antibiotic cassette followed by the native promoter of the target gene. Another, more clean strategy is to use a markerless in-frame deletion system. So far there has not been an ideal in-frame deletion system available. We have created a galactose-based in-frame deletion system for use in *S. mutans* (19). This system can be used for selected genes as the required deletion of the *galKT* genes (to facilitate selection) may obscure other phenotypes.

References

1. Klaenhammer, T. R. (1988) Bacteriocins of lactic acid bacteria. *Biochimie*. **70**, 337–349.
2. Riley, M. A., and Wertz, J. E. (2002) Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie*. **84**, 357–364.
3. Riley, M. A., and Wertz, J. E. (2002) Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol.* **56**, 117–137.
4. Sahl, H. G., and Bierbaum, G. (1998) Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu. Rev. Microbiol.* **52**, 41–79.
5. Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., and Dewhirst, F. E. (2005) Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* **43**, 5721–5732.
6. Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A., and Dewhirst, F. E. (2001) Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **183**, 3770–3783.
7. Paster, B. J., Olsen, I., Aas, J. A., and Dewhirst, F. E. (2006) The breadth of bacterial diversity in the human periodontal pocket and other oral sites. *Periodontol* 2000. **42**, 80–87.
8. Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., and Kent, R. L., Jr. (1998) Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* **25**, 134–144.
9. Hamilton, I. A. (2000) Ecological basis for dental caries, in *Oral bacterial ecology* (Kuramitsu, H. K., and Ellen, R. P., Eds.). Horizon Scientific Press, Wymondham, pp. 215–275.
10. Loesche, W. J. (1986) The identification of bacteria associated with periodontal disease and dental caries by enzymatic methods. *Oral Microbiol. Immunol.* **1**, 65–72.
11. Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., Boches, S. K., Dewhirst, F. E., and Griffen, A. L. (2002) Molecular analysis of bacterial species associated with childhood caries. *J. Clin. Microbiol.* **40**, 1001–1009.
12. Caufield, P. W., Dasanayake, A. P., Li, Y., Pan, Y., Hsu, J., and Hardin, J. M. (2000) Natural history of *Streptococcus sanguinis* in the oral cavity of infants: evidence for a discrete window of infectivity. *Infect. Immun.* **68**, 4018–4023.
13. Mikx, F. H., van der Hoeven, J. S., Plasschaert, A. J., and König, K. G. (1976) Establishment and symbiosis of *Actinomyces viscosus*, *Streptococcus sanguis* and *Streptococcus mutans* in germ-free Osborne-Mendel rats. *Caries Res.* **10**, 123–132.
14. Kreth, J., Merritt, J., Shi, W., and Qi, F. (2005) Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J. Bacteriol.* **187**, 7193–7203.
15. Qi, F., Chen, P., and Caufield, P. W. (2001) The group I strain of *Streptococcus mutans*, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. *Appl. Environ. Microbiol.* **67**, 15–21.
16. Qi, F., Chen, P., and Caufield, P. W. (2000) Purification and biochemical characterization of mutacin I from the group I strain of *Streptococcus mutans*, CH43, and genetic analysis

- of mutacin I biosynthesis genes. *Appl. Environ. Microbiol.* **66**, 3221–3229.
17. Qi, F., Chen, P., and Caufield, P. W. (1999) Purification of mutacin III from group III *Streptococcus mutans* UA787 and genetic analyses of mutacin III biosynthesis genes. *Appl. Environ. Microbiol.* **65**, 3880–3887.
 18. Podbielski, A., Spellerberg, B., Woischnik, M., Pohl, B., and Lütticken, R. (1996) Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene*. **177**, 137–147.
 19. Merritt, J., Tsang, P., Zheng, L., Shi, W., and Qi, F. (2007) Construction of a counterselection-based in-frame deletion system for genetic studies of *Streptococcus mutans*. *Oral Microbiol. Immunol.* **22**, 95–102.

Chapter 12

Natural Transformation of Oral Streptococci

Fernanda Cristina Petersen and Anne Aamdal Scheie

Abstract

Natural transformation is found in most groups of oral streptococci, including the mitis, the anginosus, and the mutans groups. This ability has been applied as a powerful tool to explore streptococcal gene functions and regulatory pathways, particularly in *Streptococcus mutans* and *Streptococcus gordonii*. The range of strains and species amenable to transformation has expanded in recent years with the identification of several competence-stimulating peptide signals (CSPs). In this chapter we present protocols for natural transformation in strains found in the three groups of transformable oral streptococci, with focus on methods using synthetic CSPs. We also include suggestions on how to optimize competence conditions for individual species or strains.

Key words: *Streptococcus*, competence, transformation, competence-stimulating peptide, CSP.

1. Introduction

Natural genetic transformation is found in most groups of oral streptococci, including the mitis, the anginosus, and the mutans groups (1). It is only in the salivarius group that transformation has not been observed. This ability has been largely applied as a powerful tool to explore streptococcal gene function and regulatory pathways. In oral streptococci, natural transformation has been particularly applied in investigations of the molecular mechanisms involved in *Streptococcus gordonii* and *Streptococcus mutans* adherence and virulence. The protocols used for transformation of oral streptococci are, therefore, based mostly on these two species. Other means to introduce exogenous DNA into oral streptococci, such as electroporation and

conjugation have also been used, but for cells exhibiting natural transformation, this is by far the simplest and most efficient approach.

Natural transformation in streptococci occurs through development of an X-state during which the cells become competent to take up DNA from the environment and to incorporate it into their chromosome (2). Competence is transient, and in liquid cultures it develops generally in early growth phase and shuts off before entering stationary phase.

The growth conditions leading to spontaneous competence development are not well defined, but subtle variations may have profound effects on the ability to develop competence (3, 4). The duration of the competent state is also influenced by growth conditions, and wide variability may be observed between different species. Optimal competence in *Streptococcus pneumoniae* growing in a liquid culture lasts, for instance, from 20 to 30 min, whereas in *S. mutans* competence may last more than 4 h (5). Protocols to transform streptococci have thus been based on ensuring that DNA is present during the predicted competence window under environmental growth conditions that have been shown to support competence development of a particular species or strain. Transformation exhibits, however, a wide range of efficiencies and strain-to-strain variability. For strains with low transformation efficiencies, optimization of the transformation protocols may thus be required.

The dependence on environmental factors to achieve efficient competence levels is in part related to the ability of the cells to produce and secrete the pheromone CSP (competence-stimulating peptide). By using synthetic CSPs, the production or secretion requirements of endogenous CSP may be bypassed, alleviating the stringency of the conditions required for spontaneous competence development. This approach has led to higher and more reproducible transformation efficiencies and extended the range of strains amenable to transformation (6).

This chapter describes transformation protocols for oral streptococci based on stimulated or spontaneous competence, which we have used to transform strains of the mutans, anginosus, and mitis groups. Variations and alternate protocols for transformation of other oral streptococci are also presented, as well as instruction on how to find the specific sequences for the synthetic CSPs used in the stimulated assays. In general, the use of competence-sustaining media and the presence of transforming DNA during the competence window are crucial for successful transformation. In addition, the use of synthetic CSPs may circumvent several of the limitations in protocols based on spontaneous competence.

2. Materials

2.1. Competence Induction Using Synthetic CSPs

1. Agar plates: Todd-Hewitt Broth (THB) 30 g/L (Difco Laboratories, Detroit, Mich). Add 15 g/L of agar to the medium and autoclave at 121°C for 15 min. For selective plates, the appropriate antibiotic(s) should be added to the medium once it has cooled to below 60°C and the media should be stored under conditions appropriate for the antibiotics (*see* **Note 1**).
2. Liquid media: For transformation of *S. mutans*, Tryptic Soy Broth (TSB; soybean-casein digest medium; Difco Laboratories) 30 g/L; for transformation of the anginosus group, Todd Hewitt Broth (THB; Difco Laboratories) supplemented with 2–10% (v/v) heat-inactivated horse serum (HS); for transformation of *Streptococcus mitis*, THB-HS and THB supplemented with 0.2% yeast extract (THY); for other oral streptococci: THY (*see* **Notes 2, 3, and 4**).
3. Synthetic peptides: Synthetic CSPs specific for the strain used may be ordered from peptide synthesis services. Stock solutions of the lyophilized peptides are prepared by resuspending the content in distilled water to a concentration of 10 mM and storing it at –20°C. Working solutions of 10 or 100 µM are aliquoted and stored at –20°C. For the CSP sequences, *see* **Section 3.4**.
4. DNA: For maximum recovery of transformants use purified DNA at final concentrations close to saturation levels (*see* **Note 5**).
5. DNaseI (Roche, DNaseI recombinant, 10 U/µL): DNaseI is used to degrade DNA not taken up by the cells. This step is optional (*see* **Note 6**).

2.2. Spontaneous Competence

1. Agar plates: THB agar prepared as described above.
2. Liquid media: THB supplemented with 2–10% heat-inactivated horse serum. Verify that the horse serum is already inactivated by the supplier. If not, inactivate the serum by incubation at 60°C for 20 min (*see* **Note 7**).

3. Methods

The appearance and the efficiency of competence development are highly dependent on environmental and growth conditions that are quite restricted and mostly not well defined. Such

conditions may influence different steps of competence including, for instance, CSP production, the expression of components in the response cascade, or shut off mechanisms. We first present protocols using synthetic CSPs, which may alleviate some of the requirements for spontaneous competence such as those influencing CSP production. The use of synthetic CSPs leads often to higher transformation efficiencies, a better control over the time of competence development, and may extend transformation to a wider range of strains. The peptides are stable and offer a straightforward approach for transformation of strains in which the CSP sequence is known. However, since the CSPs may vary among strains and species of streptococci, it may be necessary to identify the CSP sequence for strains in which the CSP sequence is unknown. Strategies that have been used for CSP identification, based on PCR amplification and sequencing, are therefore presented. We have also included an alternative approach based on spontaneous competence without synthetic CSPs. For some streptococci, transformation efficiencies obtained with this method are high enough to be used as a practical tool in transformation experiments. We also include parameters that may be adjusted to optimize the method for strains exhibiting low transformation efficiencies even in the presence of synthetic CSPs.

3.1. Transformation Efficiency/Kinetics Protocol Using Synthetic CSPs

Given the influence of (i) as yet undefined environmental conditions, (ii) the transient nature of competence, and (iii) strain-to-strain variations in transformation efficiencies, one may choose to run kinetic experiments before establishing routine transformation protocols. The following protocol has been used to determine the kinetic of transformation efficiency in *S. mutans* UA159 (*see* Fig. 12.1).

1. Stock cultures are stored at -70°C or -20°C in 15% glycerol.
2. Inoculate 5 mL TSB with $\sim 10\ \mu\text{L}$ of the stock culture using a sterile transfer loop and incubate at 37°C overnight (ON) in a 5% CO_2 (in air) atmosphere (*see* Note 8).
3. Dilute the ON culture 1:40 in TSB ($A_{600} = \sim 0.04$) and incubate at 37°C in a normal aerobic (air) atmosphere. Thaw the specific CSP on ice while waiting for the next step.
4. After 1.5–2 h incubation ($A_{600} = \sim 0.06 - 0.08$ or $\text{CFU} = \sim 7 \times 10^6/\text{mL}$), add 18-CSP to the culture to a final concentration of 50 nM and continue incubating at 37°C in air (*see* Note 9).
5. Add 50–100 ng transforming DNA to a 100 μL aliquot of the cells at different time points (*see* Fig. 12.1). Mix it by

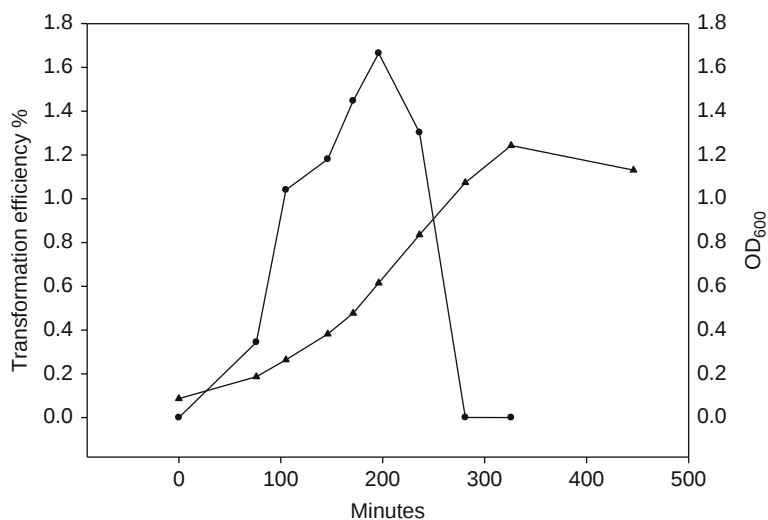


Fig. 12.1. *Streptococcus mutans* UA159 transformation efficiency in the presence of synthetic CSP during growth. The *dots* represent transformation efficiency values (with pVA838 as transforming DNA), and the *triangles* are the corresponding absorbance values at 600 nm (OD₆₀₀) of the growing culture.

gently tapping the base of the microfuge tube or gently stir with a micropipette tip. Incubate the culture at 37°C in air for 20 min (*see* **Notes 10, 11, 12, and 13**).

6. Add 200 µL pre-warmed TSB containing 20 U/mL DNase I to each aliquot of the competent cells and proceed with incubation at 37°C in air for another 40 min.
7. Spread 100–200 µL aliquots of the transformation mix on the appropriate plates supplemented with antibiotic (s). Media without antibiotic are also included for calculation of the transformation efficiency (total CFU). If necessary the cells can be diluted or concentrated prior to plating. Wait for the liquid to dry on the agar. Invert the plates and incubate them at 37°C in 5% CO₂ for 24–48 h.
8. Count the colonies in each plate and calculate the transformation efficiency.

$$\text{Transformation efficiency (\%)} = \frac{\text{CFU transformants} \times 100}{\text{total CFU}}.$$

9. Select at least three colonies (putative transformants) for further characterization. The colonies should be individually transferred to fresh TSB containing the appropriate antibiotic and incubated at 37°C in 5% CO₂ for 24 h.

10. Screen the colonies to verify whether the DNA construct was correctly incorporated. Methods based on PCR, Southern blotting, or gel electrophoresis may be used for this purpose. Follow the next two steps if the mutants will be used in downstream applications.
11. Plate the selected bacteria in the presence of the antibiotic and incubate them at 37°C in 5% CO₂ for 24–48 h. Ensure at least two passages in antibiotic-containing media (*see* **Note 14**).
12. Grow the selected bacteria in THB until reaching exponential phase and store the culture in 15% glycerol at –20°C or –70°C.

3.2. Transformation Protocols for Downstream Applications Using Synthetic CSPs

3.2.1. *Streptococcus mutans*

These are simplified protocols that we have used in experiments in which determination of the kinetics of competence are not the focus or in which acceptable efficiency levels are obtained without the need for further optimization steps.

This protocol has been used for transformation of different *S. mutans* strains (*see* **Note 15**).

1. Follow steps 1 and 2 described in **Section 3.1**.
2. Dilute the ON culture 1:40 in TSB and prepare 500–1,000 µL aliquots in 1.5 mL Eppendorf tubes. Add 18-CSP to a final concentration of 50 nM, and transforming DNA, and incubate at 37°C in air for 2.5–3 h (*see* **Notes 4 and 9**).
3. Follow the Steps 7–12 described in **Section 3.1**.

3.2.2. The Anginosus Group: *Streptococcus intermedius*, *Streptococcus anginosus*, and *Streptococcus constellatus*

This protocol, slightly modified from Gaustad and Morrison (6), has allowed transformation of *S. intermedius*, *S. anginosus*, and *S. constellatus* (*see* **Notes 4 and 16**).

1. Follow steps 1 and 2 described in **Section 3.1**, but inoculate the cells in THB-HS instead.
2. Dilute the ON culture 1:10 in THB-HS and prepare 500–1,000 µL aliquots in 1.5 mL Eppendorf tubes. Incubate at 37°C in air for 1–1.5 h. Thaw the specific CSP on ice while waiting for the next step (*see* **Note 17**).
3. Add the CSP to the culture to a final concentration of 20–50 nM and allow growing at 37°C in air for 1 h, followed by 37°C in 5% CO₂ for 1–3 h.
4. Follow steps 7–13 described above.

3.2.3. *Streptococcus mitis*

This protocol has been used for transformation of the *S. mitis*-type strain CCUG 31611. We prepare pre-cultures of *S. mitis*,

which are frozen and stored for direct use in the transformation experiments (*see* **Note 4**).

Pre-culture preparation:

1. Inoculate 5 mL THY with $\sim 10 \mu\text{L}$ of the stock culture using a sterile transfer loop and incubate at 37°C ON in 5% CO_2 aerobic atmosphere.
2. Dilute the ON culture 1:100 in THY and incubate at 37°C in 5% CO_2 aerobic atmosphere for 4–5 h $A_{600} = \sim 0.3$. Store the cells in 10% glycerol at -70°C or use it directly in the transformation experiments.

Transformation:

1. Inoculate $100 \mu\text{L}$ of the pre-culture in $900 \mu\text{L}$ THB-HS. The volumes can be scaled up but the 1:10 dilution should be maintained.
2. Add CSP at a final concentration of 200 nM and transforming DNA at the concentration described above. Incubate at 37°C in air for 3–4 h (*see* **Note 18**).
3. Follow the steps 7–12 described in **Section 3.1**.

3.2.4. Other Oral Streptococci

The protocol used for the anginosus group (**Section 3.2.2**) may also be applicable to *S. gordonii*, *S. sanguinis*, and other oral streptococci (**6**). The following modified assay that allows *S. gordonii* stocks to be stored in frozen aliquots and directly applied in competence experiments has been suggested (**7**):

Pre-culture preparation:

1. Add $10 \mu\text{g/mL}$ synthetic CSP to ON cultures grown at 37°C in THY supplemented with chloramphenicol ($5 \mu\text{g/mL}$).
2. Freeze the cells in $100 \mu\text{L}$ aliquots following the addition of 10% glycerol.

Transformation:

1. Add $900 \mu\text{L}$ THY to $100 \mu\text{L}$ of the frozen cell aliquot.
2. Add transforming DNA and incubate the cells at 37°C for 3 h.
3. Follow the steps 7–12 described in **Section 3.1**.

3.3. Spontaneous Competence

Transformation approaches based on spontaneous competence have largely been surpassed by protocols using synthetic CSPs. Nevertheless, some strains may achieve transformation efficiencies at high enough levels to allow spontaneous competence to be used as a genetic tool (*see* **Note 19**).

1. Prepare a 40-fold subculture of *S. mutans* or a 10-fold subculture of other oral streptococci in pre-warmed THB-HS from an ON THB-HS culture.

2. Incubate the culture for 1 h at 37°C in air or 5% CO₂.
3. Add transforming DNA to the culture and continue incubation for another 3 h.
4. Follow the steps 7–12 described above.

3.4. Synthetic CSPs: Sequence Identification

Verify whether the CSP for the strain you will be working with has been previously identified. Some of the CSPs that have been used for transformation of oral streptococci are presented in **Table 12.1**. For updated information on other strains, search gene/protein databases such as Entrez. If the CSP in your chosen strain has not yet been identified, you may use PCR to amplify and sequence the *comC* gene. This information is then used to predict the CSP amino acid sequence for your strain.

1. In *S. mutans*, the forward primer 5'-GCTGCGCAACC-GACATCTCTA and the reverse primer 5'-TCATCCACGACAGCACACTTGA have been used for the amplification of a 474-bp segment encompassing the *comC* gene (8). In other streptococci, the *comC* gene has been identified by using primers annealing to conserved Arg- and Glu-tRNA genes flanking the *comCDE* operon (9). In this case, the primer pair t-Arg 5'-GGCGGTGTCTTAACCCCTTGACCAACGGACC and t-Glu 5'-CATAGCTCAGCTGGATAGAGCATTCGCCTTC is expected to amplify a fragment of approximately 2.5–2.6 kb (9). The final reaction volume of 25 µL should contain 200–300 ng chromosomal DNA, Taq Polymerase or a high-fidelity DNA polymerase such as Pfu at the recommended concentration, 0.2 mM dNTP, 1× PCR buffer

Table 12.1
Sequence of CSP from type strains or genome sequenced oral streptococci in which synthetic CSP has been used in transformation

	Strain	CSP sequence	Relevant reference
Mutans group	<i>S. mutans</i> UA159 ^G	SGSLSTFFRLFNRSFTQALGK SGSLSTFFRLFNRSFTQA	(11) (8)
Mitis group	<i>S. gordonii</i> Challis CH1 ^G	DVRSNKIRLWWENIFFNKK	(17)
	<i>S. gordonii</i> NCTC 7865 ^T	DIRHRINNSIWRDIFLKRK	(18)
	<i>S. sanguinis</i> SK36 ^G	DLRGVPNPWGWFGR	(17)
	<i>S. mitis</i> NCTC 12261 ^{TG}	EIRQTHNIFNFFKRR	(19)
	<i>S. oligofermentans</i> LMG 21535 ^T	DSRNIFLKIKFKKK	(20)
Anginosus group	<i>S. anginosus</i> NCTC 10713 ^T	DSRIRMGFDFSKLFGK	(9)
	<i>S. constellatus</i> NCTC 11325 ^T	DSRIRMGFDFSKLFGK	(9)
	<i>S. intermedius</i> NCTC 11324 ^T	DSRIRMGFDFSKLFGK	(9)

T, type strain; G, genome sequence available.

containing standard MgCl_2 concentration, and $0.1 \mu\text{M}$ of forward and reverse primers. The following cycle parameters for amplification of *comC* in *S. mutans* may be used: 94°C for 3 min; 36 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and a final polymerization step of 72°C for 7 min. Adjust the PCR conditions for amplification of the *comCDE* operon in the other oral streptococci by replacing the 30 s extension time at 72°C with 2 min during the 36 cycles (*see Note 20*).

2. Verify the presence of the amplified product by electrophoresis in 0.7% (w/v) agarose gel and determine the *comC* sequence in the amplified product, which by using the primers indicated above is within ca. 250 bp from the 3'-end in *S. mutans* and ca. 350 bp from the 5'-end in other streptococci.
3. To predict the CSP sequence you may align the sequence of your amplified product with previously identified *comC* genes. Most often, the deduced CSP peptide sequence, when translated from your PCR product, is preceded by a double glycine cleavage site. Alignment of ComC with peptides of known cleavage sites may help define the mature peptide sequence (9). Many peptide synthesis services are currently available, making the acquisition of the peptides convenient and affordable.

4. Notes

1. Other suitable agar plates with media supporting growth of streptococci such as Brain Heart Infusion (BHI), TSB, or blood agar plates may also be used.
2. For transformation of *S. mutans* in the presence of CSP, the efficiency levels we obtain in TSB using the replicative plasmid pVA838 vary usually between 1 and 2%. In the *S. mutans* genome sequence reference strain UA159, these values are 10-fold higher than those obtained in THB-HS. TSB has also the advantage of supporting *S. mutans* biofilm formation, in contrast to THB-HS, thus making TSB a more suitable medium for studies investigating the association between competence and biofilm. Other media that have been used in *S. mutans* CSP transformation assays reporting efficiency levels above 0.2% include BHI-HS (10) and THY-HS (11).
3. Notice that for oral streptococci, a defined or semi-defined medium supporting transformation efficiency

levels as high as those with complex media have not yet been described. One relies therefore on non-defined media, which may to a certain degree, vary in composition according to the batch or manufacturer. We have had a previous experience in which a particular TSB batch resulted in very poor streptococcal transformation efficiencies, a problem that was solved by simply changing the batch of TSB used.

4. The influence of media, dilution of the inoculum, and other variables affecting competence in different oral streptococcal species have been mostly conducted before the introduction of synthetic CSPs. Since the use of synthetic CSPs can bypass the requirements for endogenous (natural) CSP production, it is now possible to optimize transformation conditions that favor later stages in the competence response. The increased transformation level of *S. mutans* in TSB, a medium that is poor in supporting endogenous competence in comparison with THB-HS, is an example of the potential for further optimization of transformation protocols. The possibility that the synthetic CSP-based protocols for the different oral streptococci may be interchanged without significantly affecting transformation efficiency has not been systematically investigated. But as described in **Note 2**, at least for *S. mutans* there is a clear advantage in adapting the protocols to the individual species.
5. Use specific commercial DNA purification kits for isolation of plasmids, PCR-amplified fragments, or genome DNA and follow the recommended DNA isolation protocols. Lysis procedures for oral streptococci should take into consideration the rigidity of the streptococcal cell wall. We usually incubate the cells (up to 10^9 cells) for 20 min in the presence of 100 U/mL mutanolysin and 20 mg/mL lysozyme. Purification kits are usually fast and practical to use and have been used extensively in transformation experiments. In trying alternative methods, the purity of the isolated DNA, which may impact on transformation efficiency, should be considered.
6. This step is particularly useful in kinetic studies, but may be omitted in studies in which maximal number of transformants is desirable, such as during the construction of mutants for further functional characterization. DNA uptake has been reported to be complete in *S. mutans* and *S. gordonii* between 10 and 30 min (5, 12).
7. The choice of growth medium supporting the development of spontaneous competence may be rather restricted. In

S. mutans, for instance, the use of BHI without serum supplementation or synthetic CSP, with an initial culture dilution of 1:100, results in transformation values below detection levels of 10 CFU/mL. In *S. intermedius*, TSB without CSP supplementation usually does not support competence.

8. The stock cultures may also be used to inoculate agar plates, which are incubated in 5% CO₂ for 24–48 h. In this case, one or more colonies may be used to prepare the 5 mL TSB ON culture.
9. The synthetic 18-CSP (SGSLSTFFRLFNRSFTQA) analogous to the peptide found in the supernatant of *S. mutans* induces maximal competence at 20 mM (8), but it may be used at concentrations as high as 1,000 mM, without compromising *S. mutans* transformation efficiency. Alternatively, the synthetic 21-CSP (SGSLSTFFRLFNRSFTQALGK) predicted from the *comC* sequence may be used, but maximal competence requires a concentration above 200 mM.
10. The transient nature of competence should be considered in the kinetic studies. In *S. mutans* the competent state may often last for more than 4 h (see Fig. 12.1), whereas in *S. gordonii*, for instance, it may be less than 1 h (13). The onset of competence may also vary, with *S. gordonii* exhibiting an almost immediate response to CSP (13), whereas in *S. mutans* a delay in competence induction responses are observed (10, 14).
11. Saturating levels of DNA may need to be experimentally determined. For the replicative plasmid pVA838, final concentrations of 0.5–1.0 µg/mL are usually sufficient.
12. The use of positive and negative controls is highly recommended. A negative control (without DNA added) will provide information on whether the selecting antibiotic has inhibited the growth of non-transformed cells. Positive controls are usually replicative plasmids carrying an antibiotic marker. Chromosomal DNA from antibiotic-resistant strains may also be used as a positive control. The routine use of the same positive control allows comparison of the transformation efficiency between different experiments.
13. Although streptococcal transformation protocols usually recommend growth of the cells in 5% CO₂, we usually obtain higher transformation efficiency levels when the cells are grown in air.
14. The repeated passage in media with the relevant antibiotic is conducted to avoid carry over of non-transformed

bacteria. This step is particularly pertinent when using selection markers conferring resistance to antibiotics that are bacteriostatic (i.e., those that inhibit the growth of the cells without killing them).

15. We have used this protocol to obtain higher and more reproducible transformation levels of various naturally competent *S. mutans* strains including UA159, LT11, BM71, UA130, GS-5, NG-8, and V403. The potential of extending the range of strains amenable to transformation by using synthetic CSPs has, however, not yet been addressed.
16. We have used this protocol to obtain greater and more consistent transformation levels of the *S. intermedius*, *S. anginosus*, and *S. constellatus*-type strains, and *S. intermedius* CCUG 28205. We usually observe a 10-fold increase in transformation efficiency, compared to the same protocol without the addition of synthetic CSP. Like for other oral streptococci, the potential of extending the range of strains amenable to transformation by using synthetic CSPs has not yet been examined.
17. The type strains of *S. intermedius*, *S. anginosus*, and *S. constellatus* possess the same pherotype (see **Table 12.1**). This is unlike other transformable streptococci, in which the CSPs exhibit species or even strain specificity. The extent of which the CSP of the type strains may be used for transformation of other strains in the anginosus group remains to be determined.
18. In *S. mitis* each strain usually possesses a unique CSP (15). The CSP concentration generating maximal competence levels has not been determined experimentally for *S. mitis*.
19. Typically, this protocol may be used in transformation experiments involving replicative plasmids, in which the transformation efficiency is usually a minor issue. However, in transformation experiments employing PCR-ligation mutagenesis as a strategy for gene inactivation, higher transformation efficiencies may be required (16). In this instance, the use of transformation protocols using synthetic CSPs are recommended.
20. The PCR strategy to identify the CSPs will depend on the presence of the flanking regions annealing to the specified primers. Different sequences or gene arrangements may, therefore, escape detection. Note that among the transformable oral streptococci, it is only in *S. mutans* that the *comC* gene is not flanked by the Arg- and Glu-tRNA genes (9, 11).

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References

1. Johnsborg, O., Eldholm, V., and Håvarstein, L. S. (2007) Natural genetic transformation: prevalence, mechanisms and function. *Res. Microbiol.* **158**, 767–778.
2. Claverys, J. P., Prudhomme, M., and Martin, B. (2006) Induction of competence regulons as a general response to stress in gram-positive bacteria. *Annu. Rev. Microbiol.* **60**, 451–475.
3. Shah, G. R., and Caufield, P. W. (1993) Enhanced transformation of *Streptococcus mutans* by modifications in culture conditions. *Anal. Biochem.* **214**, 343–346.
4. Morrison, D. A. (1997) Streptococcal competence for genetic transformation: regulation by peptide pheromones. *Microb. Drug Resist.* **3**, 27–37.
5. Lindler, L. E., and Macrina, F. L. (1986) Characterization of genetic transformation in *Streptococcus mutans* by using a novel high-efficiency plasmid marker rescue system. *J. Bacteriol.* **166**, 658–665.
6. Gaustad, P., and Morrison, D. A. (1998) Induction of transformation in streptococci by synthetic competence stimulating peptides. *Methods Cell Sci.* **20**, 65–70.
7. Warren, T. K., Lund, S. A., Jones, K. F., and Hruby, D. E. (2007) Comparison of transformation protocols in *Streptococcus gordonii* and evaluation of native promoter strength using a multiple-copy plasmid. *Can. J. Microbiol.* **53**, 417–426.
8. Petersen, F. C., Fimland, G., and Scheie, A. A. (2006) Purification and functional studies of a potent modified quorum-sensing peptide and a two-peptide bacteriocin in *Streptococcus mutans*. *Mol. Microbiol.* **61**, 1322–1334.
9. Håvarstein, L. S., Hakenbeck, R., and Gaustad, P. (1997) Natural competence in the genus *Streptococcus*: evidence that streptococci can change pherotype by interspecies recombinational exchanges. *J. Bacteriol.* **179**, 6589–6594.
10. Ahn, S. J., Wen, Z. T., and Burne, R. A. (2006) Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. *Infect. Immun.* **74**, 1631–1642.
11. Li, Y. H., Lau, P. C., Lee, J. H., Ellen, R. P., and Cvitkovitch, D. G. (2001) Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *J. Bacteriol.* **183**, 897–908.
12. Murchison, H. H., Barrett, J. F., Cardineau, G. A., and Curtiss, R., 3rd. (1986) Transformation of *Streptococcus mutans* with chromosomal and shuttle plasmid (pYA629) DNAs. *Infect. Immun.* **54**, 273–282.
13. Vickerman, M. M., Iobst, S., Jesionowski, A. M., and Gill, S. R. (2007) Genome-wide transcriptional changes in *Streptococcus gordonii* in response to competence signaling peptide. *J. Bacteriol.* **189**, 7799–7807.
14. Kreth, J., Merritt, J., Shi, W., and Qi, F. (2005) Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Mol. Microbiol.* **57**, 392–404.
15. Kilian, M., Poulsen, K., Blomqvist, T., Håvarstein, L. S., Bek-Thomsen, M., Tetelín, H., and Sørensen, U. B. (2008) Evolution of *Streptococcus pneumoniae* and its close commensal relatives, PLoS One. **3**, e2683.
16. Lau, P. C., Sung, C. K., Lee, J. H., Morrison, D. A., and Cvitkovitch, D. G. (2002) PCR ligation mutagenesis in transformable streptococci: application and efficiency. *J. Microbiol. Methods.* **49**, 193–205.
17. Gaustad, P., and Håvarstein, L. S. (1997) Competence-pheromone in *Streptococcus sanguis*, in *Streptococci and the host* (Horáud, T., Bouvet, A., Leclercq, R., de Montclos, H., and Sicard, M., Eds.). Plenum Press, New York, pp. 1019–1021.

18. Håvarstein, L. S., Gaustad, P., Nes, I. F., and Morrison, D. A. (1996) Identification of the streptococcal competence-pheromone receptor. *Mol. Microbiol.* **21**, 863–869.
19. Johnsborg, O., Eldholm, V., Bjornstad, M. L., and Håvarstein, L. S. (2008) A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species. *Mol. Microbiol.* **69**, 245–253.
20. Tong, H., Zhu, B., Chen, W., Qi, F., Shi, W., and Dong, X. (2006) Establishing a genetic system for ecological studies of *Streptococcus oligofermentans*. *FEMS Microbiol. Lett.* **264**, 213–219.

Chapter 13

Use of *In Vivo*-Induced Antigen Technology (IVIAT) to Identify Virulence Factors of *Porphyromonas gingivalis*

Shannon M. Wallet, Jin Chung, and Martin Handfield

Abstract

Porphyromonas gingivalis is a Gram-negative anaerobic bacterium associated with the initiation and progression of adult periodontal disease. The pathogenicity of *P. gingivalis* is multifaceted and the infection process is influenced by both microbial and host factors. It is generally accepted that genes of a pathogen that are specifically expressed during infection are likely to be important for pathogenicity. Numerous technologies have been developed to identify these genes. A novel strategy known as *in vivo*-induced antigen technology (IVIAT) avoids the use of animal models and utilizes serum from patients who have experienced disease caused by the pathogen of interest. While a number of putative virulence factors have been described for *P. gingivalis*, the identity, relevance, and mechanisms of action of virulence factors that actually provide a selective advantage to the organism in the oral cavity of diseased patients is still unclear. Here we describe the IVIAT protocol for identification of *in vivo*-induced genes of *P. gingivalis*, which can be adapted with few modifications to any microbial pathogen.

Key words: *Porphyromonas gingivalis*, *in vivo*-induced antigen technology (IVIAT), virulence, pathogenesis, sequencing, genomic expression library, enzyme-linked immunosorbent assay (ELISA), absorption, proteomic, genomic, signature-tagged mutagenesis (STM), differential fluorescence induction (DFI), *in vivo* expression technology (IVET).

1. Introduction

The pathogenesis of a microbial infection in humans is a complex and dynamic process, constantly evolving within the host. In addition, production of virulence determinants is tightly regulated and modulated in response to the ever-changing environment encountered at the site of infection. *Porphyromonas gingivalis* is a Gram-negative anaerobic bacterium associated with the initiation and progression of human adult periodontal disease (1, 2). The pathogenicity of *P. gingivalis* is multifaceted and the

infection process is influenced by both microbial and host (immunological) factors. While a number of putative virulence factors have been described for *P. gingivalis*, the identity, relevance, and mechanisms of action (function) of virulence factors that actually provide a selective advantage to the organism in the oral cavity of a diseased patient are still unclear (3–5).

Obviously, all regulated virulence determinants of a pathogen such as *P. gingivalis* cannot be identified *in vitro*, because of the technically impossible task of mimicking all the different environmental stimuli that occur at the site of infection. Therefore, this can hamper our understanding of the virulence mechanisms of the pathogen of interest. To overcome this problem, we must emphasize the need to study bacterial virulence using organisms engaged in an actual infectious process. Several methods such as *in vivo* expression technology (IVET), signature-tagged mutagenesis (STM), differential fluorescence induction (DFI), and microarray analysis have been devised to accomplish this task (6, 7). While the methods developed to date have contributed significantly to our understanding of bacterial pathogenesis, they all suffer from important drawbacks. Most notably and in most instances, they depend on the use of animal models to monitor the pathogen growing in an actual site of infection. Frequently, the animal model does not closely resemble, mimic, or even reflect the conditions found within the natural human host. Often, there simply is no suitable animal model for a particular pathogen, as in the case of *P. gingivalis*. Recently, the design of the novel method IVIAT has been used to study microbial pathogenesis and accomplishes the same goals as IVET, STM, and DFI, yet does not require the use of potentially misleading animal models (8). To date, IVIAT has successfully been used to study over 33 different prokaryotic and eukaryotic pathogens, including *P. gingivalis* and uncovered >1,000 *in vivo*-induced antigens (3, 5, 7, 9–17). More importantly, this approach is a non-invasive method of evaluating bacterial pathogenesis within a human host. Therefore, we can study host genetics as well as host environmental influences on *in vivo*-induced gene expression of bacteria.

A general overview of the IVIAT scheme is presented in **Fig. 13.1** (adapted from (18)). Serum from patients who have experienced an infection caused by the pathogen under study is pooled and repeatedly adsorbed with *in vitro* grown cells of the pathogen leaving antibodies against antigens that are expressed only *in vivo*. A genomic expression library of the pathogen's DNA is generated in a suitable host and clones are probed with the adsorbed serum. Reactive clones, which presumably are producing antigens expressed during a natural infection but which are not expressed during *in vitro* cultivation are purified and their cloned DNA sequenced. Genes are identified in this fashion as encoding *in vivo*-induced (IVI) antigens. These antigens are purified and used to verify that the IVI antigen is expressed

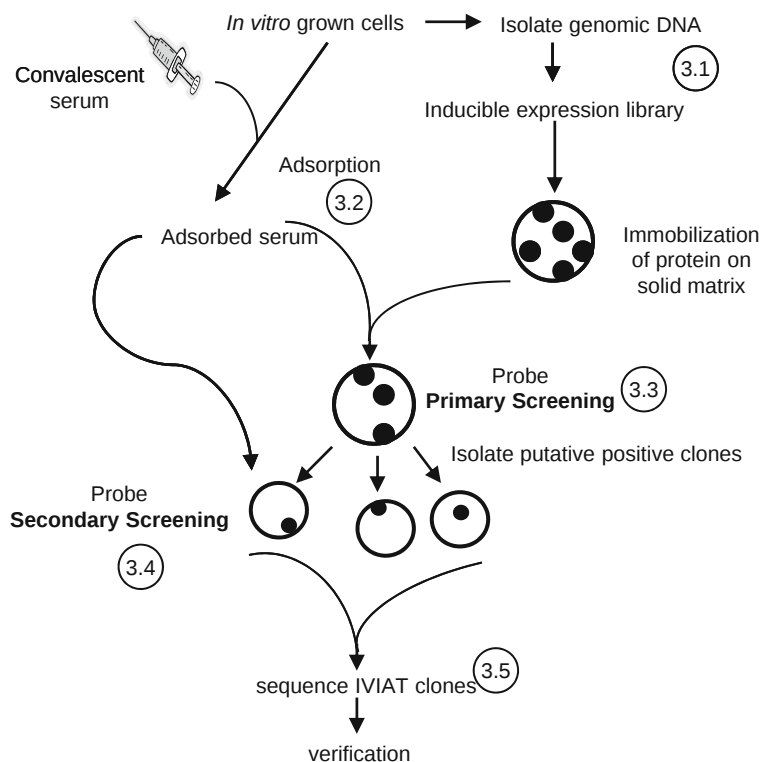


Fig. 13.1. The IVIAT system. A general overview of the IVIAT scheme is presented here. Numbers in the *circles* indicate sections of the chapter in which detailed protocols can be found. (3.1) A genomic expression library of *P. gingivalis* ATCC 33277 is generated in *E. coli* using the pET-30 expression system. (3.2) Serum from patients with gingivitis is pooled and repeatedly adsorbed with *in vitro* grown cells of *P. gingivalis* ATCC 33277. (3.3) The expression library is probed with adsorbed serum (primary screening). (3.4) Reactive clones (putative positive clones) are isolated and re-probed with adsorbed serum (secondary screening). (3.5) Recombinant plasmids from reactive clones from both screening steps, i.e., the IVI clones, are purified and their cloned DNA (inserts) sequenced. A final verification step is usually performed depending on the experimental design.

by the pathogen during an infectious process. This can be done in various ways but will not be discussed in detail here. Here, we describe the IVIAT protocol for identification of *in vivo*-induced genes of *P. gingivalis*.

2. Materials

2.1. Construction of a Genomic Expression Library in *Escherichia coli*

1. QIAGEN® Genomic-tip Kit 500 (Qiagen, Valenica, CA)
2. Hydroshear® (Genomic® Solutions, Ann Arbor, MI)
3. GeneClean® Turbo (Q-BIOgene, Carlsbad, CA)
4. End-It™ DNA end-repair kit (Epicenter® Biotechnologies, Madison, WI).

5. Quick LigationTM Kit (New England Biolabs, Ipswich, MA)
6. *E. coli* BL21(λ DE3) electrocompetent cells (Stratagene, La Jolla, CA)
7. pET-30abc system (Novagen, Madison, WI)
8. *EcoRV* and complimentary NEB buffer (New England Biolabs, Ipswich, MA)
9. Calf intestinal phosphatase (CIP) (New England Biolabs, Ipswich, MA)
10. Tris-acetate EDTA (TAE) buffer (1 $\times$): 40 mM Tris-HCl, 1 mM EDTA, dilute from 50 $\times$ stock solution (242 g Tris base, 37.2 g Na₂-EDTA, 57.1 mL glacial acetic acid).
11. NuSieve GTG agarose (FMC BioProducts, Rockland, ME).
12. 0.75% GTG agarose gel: 0.75 g of GTG agarose per 100 mL of 1 $\times$ TAE.
13. DNA molecular weight markers
14. Phenol-chloroform and chloroform
15. Brain Heart Infusion (BHI) agar plates containing 50 μ g/mL of kanamycin: 37 g/L broth powder (Becton Dickinson & Co.), 20 g/L agar. Sterilize by autoclaving for 20 min at 121°C. Cool to 50–55°C before dispensing into petri dishes. If other chemicals, e.g., kanamycin, are required, they must be added to the cooled agar immediately prior to pouring the agar.
16. Kanamycin: 50 mg/mL stock (dissolved in sterile deionized water). *Optional* – Filter-sterilize (0.22 μ m pore size membrane filter).
17. Glycerol
18. Clean scalpel.

2.2. Adsorption of Target Serum

1. Convalescent sera from experimental population of choice.
2. *Porphyromonas gingivalis* ATCC 33277 grown anaerobically at 37°C in TSBYE medium [Trypticase Soy Broth supplemented with 0.6% yeast extract, 5 μ g/mL hemin, and 1 μ g/mL menadione (vitamin K)].
3. *E. coli* host strain BL21(λ DE3) grown aerobically in LB broth (per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, pH 7.0) at 37°C.
4. Phosphate-buffered saline (PBS).
5. PBS + 0.02% sodium azide.
6. PBS containing 0.1% Tween-20 (PBS/Tween)

7. Bicarbonate-coating buffer: 5.3 g/L Na₂CO₃, 4.2 g/L NaHCO₃, 1 g/L sodium azide, pH 9.6.
8. Peroxidase-conjugated goat anti-human immunoglobulin (Cappel/ICN, Aurora, OH)
9. *p*-Nitrophenyl phosphate liquid substrate (pNP reagent) (Sigma, St. Louis, MO)
10. 3 M NaOH
11. 82 mm diameter (45 μm pore size) nitrocellulose membranes (GE Osmonics, Minnetonka, MN)
12. Petri dishes
13. 96-well microtiter plates
14. Boiling water bath
15. French pressure cell press (French press)
16. Spectrophotometer with plate reading capabilities

2.3. Primary Screening of Genomic Expression Library

1. Unadsorbed and/or adsorbed infected and/or convalescent sera from experimental population of choice
2. 1 M stock of isopropylthio-D-galactopyranoside (IPTG): 0.2338 g IPTG in 10 mL sterile deionized water. Dispense into 1 mL aliquots and store at -20°C.
3. Kanamycin (50 mg/mL stock solution in deionized water).
4. BHI broth + 50 μg/L kanamycin (BHI/Kan).
5. BHI agar + 50 μg/L kanamycin (BHIA/Kan).
6. BHI agar + 50 μg/L kanamycin + 1 mM IPTG (BHIA/Kan/IPTG).
7. PBS with 0.1% Tween-20 (PBS/Tween).
8. Bicarbonate-coating buffer: 5.3 g/L Na₂CO₃, 4.2 g/L NaHCO₃, 1 g/L sodium azide, pH 9.6.
9. 5% nonfat skim milk in PBS/Tween.
10. Horseradish peroxidase-conjugated goat anti-human immunoglobulin (Cappel-ICN, Aurora, OH).
11. Enhanced Chemiluminescence (ECL) detection kit and Hyperfilm (both supplied by Amersham, Piscataway, NJ).
12. Chloroform in a hermetic container.
13. Nitrocellulose membranes (82 mm diameter, 45 μm pore size) (Gibco/BRL).

2.4. Secondary Screening of Putative Positive Clones

1. Adsorbed convalescent sera from experimental population of choice.
2. Items 2–13 listed in **Section 2.3**.
3. Spotting template.

2.5. Sequencing and Characterization of IVIAT Clones

1. QIAGEN® Tip-500 plasmid isolation kit (Qiagen, Valenica, Ca)
2. pET-30 primers (Novagen, Madison, WI)
3. pET-30abc system (Novagen, Madison, WI).
4. *E. coli* BL21(λDE3) electrocompetent cells (Stratagene, La Jolla, CA).
5. pDRAW DNA analysis software (<http://www.acaclone.com>).
6. PEDANT database (<http://pedant.gsf.de/index.jsp>).
7. MacVector v.6.0.1 DNA sequence analysis package (<http://www.macvector.com>).
8. PBS with 0.1% Tween-20 (PBS/Tween).
9. Bicarbonate-coating buffer: 5.3 g/L Na₂CO₃, 4.2 g/L NaHCO₃, 1 g/L sodium azide, pH 9.6.
10. Peroxidase-conjugated goat anti-human immunoglobulin (Cappel-ICN, Aurora, Ohio).
11. *p*-Nitrophenyl phosphate liquid substrate (pNP reagent) (Sigma, St. Louis, MO).
12. 3 M NaOH.

3. Methods

3.1. Construction of a Genomic Expression Library in *E. coli*

Library construction utilizes the pET-30abc system and *E. coli* BL21(λDE3) electrocompetent cells as previously described (19). Other expression systems can be used to provide a comparable expression platform, but would require optimization.

1. Genomic DNA from *Porphyromonas gingivalis* ATCC 33277 is purified using a QIAGEN® Genomic-tip Kit 500 following the manufacturer's instructions.
2. Genomic DNA is treated in a Hydroshear® under conditions tested empirically to generate fragments of 0.5–1.5 kb fragments (*see* **Notes 1** and **2**).
3. Fragmented DNA is separated by electrophoresis on low-melting NuSieve GTG agarose in Tris–acetate EDTA (TAE). A 0.75% agarose gel is used.
4. DNA fragments are excised using a clean scalpel blade. Long wavelength UV transillumination is used to visualize and identify gel fragments of interest as indicated by appropriate molecular weight markers (*see* **Note 3**).
5. DNA fragments are purified from the agarose gel slices using GeneClean® Turbo (or equivalent, e.g., Qiagen Qiaquick Gel Extraction kit).

6. Terminal overhangs are removed using the End-It[®] DNA end-repair kit.
7. pET-30a, -30b, and -30c DNA are individually digested with *EcoRV* and treated with calf intestinal phosphatase (CIP) according to the manufacturer's instructions.
8. The resulting blunt-ended products from step 6 (above) are ligated into the CIP-treated *EcoRV* digested pET-30a, -30b, -30c multiple cloning sites to create three separate genomic libraries (*see* **Notes 4** and **5**).
9. The vector library is amplified by electroporating the ligation mixture into *E. coli* BL21(λ DE3) electrocompetent cells.
10. Transformants are selected on BHI agar plus 50 μ g/mL kanamycin (BHIA/Kan) plates.
11. After overnight incubation at 37°C, the plates are scraped to collect the colonies which are stored frozen in BHI broth containing 50% glycerol at -80°C.

3.2. Adsorption of Target Serum

A good clinical history for every serum sample is essential in the interpretation of IVIAT data. The more serum samples which can be obtained from patients representing different routes of infection and/or different stages of infection, the better the chances of recovering the broadest array of *in vivo*-induced genes (19). Serum and/or plasma from appropriate patients and controls can be used for probing the genomic library. Pooled serum is adsorbed to remove antibodies that are reactive with proteins made by the pathogen during *in vitro* cultivation as well as reactivity against the expression vector host *E. coli* BL21(λ DE3).

1. Equal amounts of each serum from a given experimental group are pooled.
2. 500 μ L of pooled serum is subjected to five successive direct adsorptions with *P. gingivalis* ATCC 33277 grown in supplemented TSBYE broth at 37°C under anaerobic conditions (*see* **Note 6**).
3. Each adsorption consists of an overnight incubation with mild agitation at 4°C of the pooled serum with $\sim 10^{11}$ bacteria in 100 mL PBS/0.02% sodium azide.
4. Bacteria are removed by centrifugation in a microfuge for 2 min at 4°C, after which the supernatant (serum) is recovered and used for the next round of adsorption. Take a small aliquot (100 μ L) for ELISA (*see* below).
5. The serum is further adsorbed by exposing it to a nitrocellulose membrane saturated with extracts of the pathogen prepared by French press treatment as follows:

- a. $\sim 10^{11}$ bacteria are suspended in 1 mL of PBS/0.02% sodium azide and treated at 14,000 psi in a French pressure cell.
- b. The entire volume of the extract is incubated for 1 h with a nitrocellulose membrane at room temperature with gentle agitation.
- c. The membrane is washed three times with PBS/Tween.
- d. Coated membranes can be kept for 1 week in PBS/Tween at 4°C until ready for use.
6. One milliliter (1 mL) of pooled serum is applied directly to the membrane in a Petri dish and incubated overnight at 4°C with gentle agitation.
7. The serum is recovered by draining the membrane and washing it with 500 μ L of PBS. Take a small aliquot (100 μ L) for ELISA (*see below*).
8. The aspirated sample and wash are pooled.
9. An additional adsorption step is carried out using the same French press cell extract prepared in step 5 (*see above*) except that the French press extract is heat denatured in a boiling water bath for 10 min before immobilization on a nitrocellulose membrane (*see Note 7*).
10. The serum is recovered by draining the membrane and washing it with 500 mL of PBS. Take a small aliquot (100 μ L) for ELISA (*see below*).
11. The aspirated sample and wash are pooled.
12. Adsorption of the pooled serum will also be performed using the host strain *E. coli* BL21(λ DE3) grown in LB broth at 37°C (*see Note 6*), repeating steps 9–11 (*see Note 8*).
13. An ELISA procedure is used to test the efficacy of the adsorption against both *in vitro* grown *P. gingivalis* ATCC 33277 and the expression vector host *E. coli* as follows:
 - a. French press extracts are immobilized in microtiter wells by incubating in bicarbonate-coating buffer at 4°C overnight.
 - b. Serial dilutions of serum samples taken at different points in the adsorption process (steps 4, 7, and 10, *see above*) are reacted with the immobilized extracts for 1 h at room temperature with mild agitation.
 - c. Microtiter wells are washed three times with 200 μ L of PBS/Tween.
 - d. 100 μ L of peroxidase-conjugated goat anti-human immunoglobulin at a 1:5,000 dilution in PBS/Tween

is incubated at room temperature for 1 h with mild agitation.

- e. Microtiter wells are washed three times with 200 μL of PBS/Tween.
 - f. 100 μL of *p*-nitrophenyl phosphate substrate (pNPP) reagent is added to each well.
 - g. Development of a yellow color can take from 5 to 30 min.
 - h. Stop the reaction with 100 μL of 3 M NaOH per well.
 - i. Read reaction on spectrophotometer at 450 nm (OD_{405}).
14. The resulting adsorbed serum is aliquoted and stored at -80°C .

3.3. Primary Screening of Genomic Expression Library

Once pooled serum has been adsorbed and demonstrates decreased reactivity to *in vitro* grown *P. gingivalis* ATCC 33277 and the expression vector host *E. coli*, initial screening of the genomic library can be performed. Performing this step prior to efficient adsorption will result in false positives when trying to identify IVI antigens.

1. The established genomic library (*see* **Section 3.1**) is thawed and serial dilutions are spread on BHIA/Kan plates such that approximately 500 colonies are obtained.
2. The optimal dilution is then plated onto BHIA/Kan and allowed to incubate for 12–14 h at 37°C to allow for optimal growth.
3. These plates are then replicated using nitrocellulose membranes onto duplicate BHIA/Kan/IPTG [BHI agar containing 50 $\mu\text{g}/\text{mL}$ kanamycin and 1 mM isopropyl- β -D-1 thiogalactopyranoside (IPTG)] and incubated for 5 h at 37°C to induce expression of the cloned genes.
4. Colonies are then lifted with nitrocellulose membranes. It is important to mark the master agar plate and nitrocellulose membrane for later identification of reactive colonies (*see* **Fig. 13.3**).
5. Lifted colonies are exposed to chloroform vapors for 15 min in a hermetic container (*see* **Note 9**) to partially lyse the bacteria and expose induced proteins.
6. Membranes are then saturated with 5% nonfat skim milk in PBS with 0.1% Tween-20 (PBS/Tween) and allowed to incubate for 1 h with mild agitation (rocking) at room temperature.
7. Membranes are washed three times in PBS/Tween for 5 min with gentle agitation at room temperature.

8. After which, membranes are incubated with unadsorbed and/or adsorbed sera (*see Note 10*) using a 1:5,000 to 1:10,000 dilution in PBS/Tween (*see Note 11*). The membranes are incubated at room temperature for 2 h with mild agitation in a final volume of 10 mL.
9. Membranes are washed three times in PBS/Tween for 5 min with gentle agitation at room temperature.
10. Membranes are incubated with peroxidase-conjugated goat anti-human immunoglobulin at a 1:5,000 dilution in PBS/Tween. Membranes are incubated at room temperature for 1 h with mild agitation at room temperature.
11. Membranes are washed three times in PBS/Tween for 5 min with gentle agitation at room temperature.
12. Serum reactivity is visualized using the ECL kit and Hyperfilm ECL from Amersham following manufacturer's direction (*see Note 12*).
13. Clones which reacted with adsorbed serum (**Fig. 13.2**) (these will be referred to as putative positive clones) are identified by their position on the master plates and purified on BHI agar plates containing 50 $\mu\text{g}/\text{mL}$ of kanamycin.

3.4. Secondary Screening of Putative Positive Clones

A secondary screen of isolated clones is used to verify reactivity by serum of interest. This step is particularly important if there were neighboring colonies in the primary screening (**Fig. 13.2**). Testing of positive clones using the secondary screening method should be repeated at least twice.

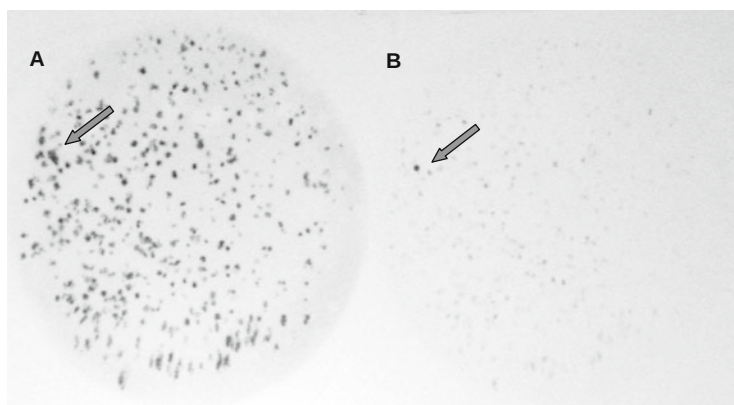


Fig. 13.2. Adsorption technique. An optimal serial dilution of the *P. gingivalis* ATCC 33277 genomic library was plated onto BHI/Kan agar plates containing 1 mM of IPTG. Then, duplicate membranes were lifted and reacted with pooled unadsorbed (**A**) or adsorbed (**B**) sera and visualized by chemiluminescence. Arrows indicate a putative positive clone which reacts with adsorbed serum (**B**), indicating expression of an *in vivo*-induced antigen by this clone. Secondary screening was subsequently performed to confirm reactivity.

1. Inoculate 1 mL of BHI/Kan in a 1.5 mL microfuge tube with a portion of a single colony of a putative positive clone from step 13 of **Section 3.3** and incubate overnight at 37°C standing.
2. Centrifuge the culture in a microfuge at top speed for 5 min at room temperature.
3. Decant supernatant and resuspend pellet in 20 mL BHI/Kan by vigorous vortexing (*see Note 13*).
4. Spot 1 mL of sample onto BHI/Kan agar plates containing 1 mM of IPTG using spotting template (**Fig. 13.3**) and incubate 5 h at 37°C.

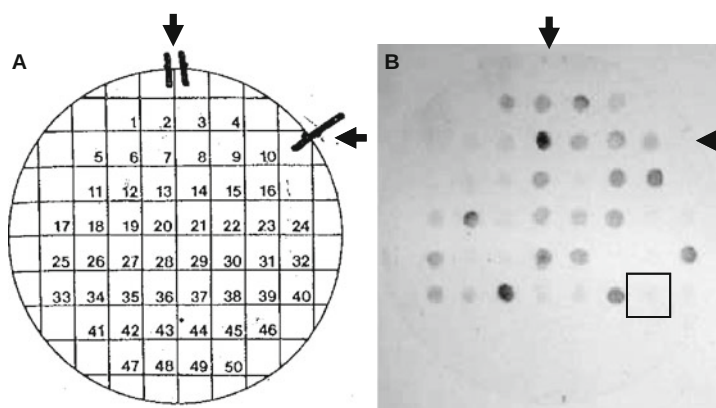


Fig. 13.3. Secondary screening technique. Colonies were spotted using spotting template (**A**) onto BHI/Kan agar plates (**B**) containing 1 mM of IPTG. Afterward, they were lifted using nitrocellulose membranes which were marked for orientation (*arrows*) during identification of reactive colonies. Colonies were then reacted with adsorbed pooled sera and reactivity was detected by chemiluminescence. The negative control consisted of the pET-30 expression vector without any DNA insert (*square*).

5. Colonies are then lifted with nitrocellulose membranes. It is important to mark the master agar plate and nitrocellulose membrane for later identification of reactive colonies (**Fig. 13.3**).
6. Lifted colonies are exposed to chloroform vapors for 15 min in a hermetic container (*see Note 9*) to partially lyse the bacteria and expose induced proteins.
7. Membranes are then saturated with 5% nonfat skim milk in PBS with 0.1% Tween-20 (PBS/Tween) and allowed to incubate for 1 h with mild agitation at room temperature.
8. Membranes are washed three times in PBS/Tween for 5 min with gentle agitation at room temperature.
9. After which, membranes are incubated with adsorbed sera from step 3.2.14 using a 1:5,000 to 1:10,000 dilution in PBS/Tween (*see Note 11*). The membranes are incubated

at room temperature for 2 h with mild agitation in a final volume of 10 mL.

10. Membranes are washed three times in PBS/Tween for 5 min with gentle agitation at room temperature.
11. Membranes are incubated with peroxidase-conjugated goat anti-human immunoglobulin at a 1:5,000 dilution in PBS/Tween. Membranes are incubated at room temperature for 1 h with mild agitation at room temperature.
12. Membranes are washed three times in PBS/Tween for 5 min with gentle agitation at room temperature.
13. Serum reactivity is visualized using the ECL kit and Hyperfilm following manufacturer's instructions (*see* **Note 12**).
14. Clones which are reactive only to adsorbed serum using the secondary screening method (these will be referred to as IVIAT clones) will be expanded and stored in BHI broth containing 50% glycerol at -80°C .

3.5. Sequencing and Characterization of IVIAT Clones

Sequencing data will disclose whether a contiguous fusion protein relative to the pET leader sequence is being expressed. Antigens may also be expressed from native ribosome-binding sites or promoters that are recognized in *E. coli*. Genes of proteins expressed from native regulatory elements may be in either orientation on cloned DNA. For these reasons, a definitive demonstration of the identity of an IVI antigen may require that all gene identified within the inserted DNA be subcloned and their products evaluated for immunoreactivity using an ELISA or a Western Blot, for example.

1. Vector DNA from the reactive clones identified in step 14 of **Section 3.4** (*see* above) is purified using a Qiagen[®] Tip-500 plasmid isolation kit.
2. The DNA inserts are sequenced (in both directions) according to the manufacturer's protocol using pET-30 primers.
3. Forward and reverse sequences are aligned using pDRAW DNA analysis software.
4. Complete insert sequences are blasted against the entire genome of *P. gingivalis* ATCC 33277 using the PEDANT (or any other publicly available) database to identify *P. gingivalis* specific open reading frames.
5. MacVector v.6.0.1 and/or pDRAW are used to determine directionality and location of all identified ORFs within the random gDNA insert.
6. All potential ORFs are subcloned using the pET-30abc system and *E. coli* BL21(λ DE3) electrocompetent cells as previously described ([19](#)).

7. Reactivity to protein products of subcloned ORFs is quantitatively estimated using an ELISA protocol:
 - a. Purified protein products (*see* **Note 14**) of subcloned IVIAT genes are immobilized in microtiter wells using overnight incubation in bicarbonate-coating buffer at 4°C.
 - b. Serial dilutions of adsorbed serum samples are reacted with the immobilized antigens for 1 h at room temperature with mild agitation.
 - c. Microtiter wells are washed three times with 200 mL of PBS/Tween.
 - d. 100 mL of peroxidase-conjugated goat anti-human immunoglobulin at a 1:5,000 dilution in PBS/Tween is incubated at room temperature for 1 h with mild agitation.
 - e. Microtiter wells are washed three times with 200 µL of PBS/Tween.
 - f. 100 µL of pNP reagent is added to each well.
 - g. Development can take anywhere from 5 to 30 min.
 - h. Stop the reaction with 100 µL of 3 M NaOH per well.
 - i. Read the reaction using spectrophotometer at OD 405.

3.6. Confirmation of Protein Expression During Human Expression

Our protocol for isolation of an *in vivo*-induced antigen using adsorbed serum does not eliminate the possibility that the reactive antibodies were originally raised against a cross-reacting protein. While confirmatory testing of expression during human infection is optional, it would discount this possibility before investing significant time and resources in the analysis of a particular IVIAT protein. There are several possible approaches that can be used to independently confirm that a protein discovered by IVIAT is actually expressed by the pathogen during a human infection and as such is beyond the scope of this chapter. One such approach includes immunofluorescent detection in infected human tissues utilizing monoclonal antibodies raised in mice, rabbits, or guinea pigs (19).

4. Notes

1. The approach described above avoids the bias created by using restriction enzymes.
2. Once the optimal conditions for digestion are found, the digestion is typically scaled up to use approximately 20 µg of starting genomic DNA.

3. Try fitting in as much DNA as possible in the fewest number of tracts to limit the amount of agarose subsequently requiring digestion. Avoid short wavelength UV light to illuminate DNA since this is likely to introduce mutations.
4. Ligation reactions are empirical, but reactions conditions should be varied in order to achieve a minimum of 5,000 independent clones per reaction.
5. Utilization of all three vectors allows for DNA to be inserted into three reading frames with appropriate regulatory signals downstream of the IPTG promoter. Other expression systems may also be used (10).
6. For these steps, the pathogen of interest is typically grown in a rich medium such as TSBYE.
7. This step allows for exposure of additional linear immunoreactive epitopes.
8. The necessity of this step in large part depends on the reactivity of the serum to *E. coli*, which may vary from subject to subject, but we routinely use this step to decrease the background observed in the screening steps to follow.
9. A dessicator with chloroform-saturated paper towels has successfully been used.
10. This step is *optional*, but it is gratifying to see that most of the clones react with unadsorbed serum, indicating that the absorption process actually eliminated antibodies directed against *in vitro*-induced genes (Fig. 13.3).
11. Obviously the dilution depends on the titer of the starting serum, but a 1:5,000 dilution is routinely utilized.
12. The conditions for probing with the primary and secondary antibodies are optimized for use of these ECL kits, therefore, adjustments may be required if other labeling methods are used. The chemiluminescent method provides greater sensitivity and lower background than colorimetric peroxidase assays.
13. Often the volume remaining in the microfuge tube after centrifugation is sufficient.
14. There are a multitude of methods for purifying proteins expressed in *E. coli* under the direction of the pET-30abc expression vector. In this particular instance, the hexahistidine (His₆)-tag has been successfully used to purify protein fusions by nickel column affinity chromatography (also refer Chapter 21 by Tabeta and Yamazaki, this volume).

References

1. Lamont, R. J., and Jenkinson, H. F. (1998) Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev.* **62**, 1244–1263.
2. Holt, S. C., Kesavalu, L., Walker, S., and Genco, C. A. (1999) Virulence factors of *Porphyromonas gingivalis*. *Periodontol* **2000**, **20**, 168–238.
3. Cao, S. L., Progulske-Fox, A., Hillman, J. D., and Handfield, M. (2004) In vivo induced antigenic determinants of *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol. Lett.* **237**, 97–103.
4. Richardson, J., Craighead, J. C., Cao, S. L., and Handfield, M. (2005) Concurrence between the gene expression pattern of *Actinobacillus actinomycetemcomitans* in localized aggressive periodontitis and in human epithelial cells. *J. Med. Microbiol.* **54**, 497–504.
5. Song, Y. H., Kozarov, E. V., Walters, S. M., Cao, S. L., Handfield, M., Hillman, J. D., and Progulske-Fox, A. (2002) Genes of periodontopathogens expressed during human disease. *Ann. Periodontol.* **7**, 38–42.
6. Davey, M. E., and Costerton, J. W. (2006) Molecular genetics analyses of biofilm formation in oral isolates. *Periodontol* **2000**, **42**, 13–26.
7. Handfield, M., Progulske-Fox, A., and Hillman, J. D. (2005) In vivo induced genes in human diseases. *Periodontol* **2000**, **38**, 123–134.
8. Duncan, M. J. (2005) Oral microbiology and genomics. *Periodontol* **2000**, **38**, 63–71.
9. Rollins, S. M., Peppercorn, A., Hang, L., Hillman, J. D., Calderwood, S. B., Handfield, M., and Ryan, E. T. (2005) In vivo induced antigen technology (IVIAT). *Cell Microbiol.* **7**, 1–9.
10. Deb, D. K., Dahiya, P., Srivastava, K. K., Srivastava, R., and Srivastava, B. S. (2002) Selective identification of new therapeutic targets of *Mycobacterium tuberculosis* by IVIAT approach. *Tuberculosis (Edinb.)* **82**, 175–182.
11. Hang, L., John, M., Asaduzzaman, M., Bridges, E. A., Vanderspurt, C., Kirn, T. J., Taylor, R. K., Hillman, J. D., Progulske-Fox, A., Handfield, M., Ryan, E. T., and Calderwood, S. B. (2003) Use of *in vivo*-induced antigen technology (IVIAT) to identify genes uniquely expressed during human infection with *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA*. **100**, 8508–8513.
12. Harris, J. B., Baresch-Bernal, A., Rollins, S. M., Alam, A., LaRocque, R. C., Bikowski, M., Peppercorn, A. F., Handfield, M., Hillman, J. D., Qadri, F., Calderwood, S. B., Hohmann, E., Breiman, R. F., Brooks, W. A., and Ryan, E. T. (2006) Identification of *in vivo*-induced bacterial protein antigens during human infection with *Salmonella enterica* serovar Typhi. *Infect. Immun.* **74**, 5161–5168.
13. John, M., Kudva, I. T., Griffin, R. W., Dodson, A. W., McManus, B., Krastins, B., Sarracino, D., Progulske-Fox, A., Hillman, J. D., Handfield, M., Tarr, P. I., and Calderwood, S. B. (2005) Use of *in vivo*-induced antigen technology for identification of *Escherichia coli* O157:H7 proteins expressed during human infection. *Infect. Immun.* **73**, 2665–2679.
14. Kim, Y. R., Lee, S. E., Kim, C. M., Kim, S. Y., Shin, E. K., Shin, D. H., Chung, S. S., Choy, H. E., Progulske-Fox, A., Hillman, J. D., Handfield, M., and Rhee, J. H. (2003) Characterization and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. *Infect. Immun.* **71**, 5461–5471.
15. Salim, K. Y., Cvitkovitch, D. G., Chang, P., Bast, D. J., Handfield, M., Hillman, J. D., and de Azavedo, J. C. (2005) Identification of group A *Streptococcus* antigenic determinants upregulated *in vivo*. *Infect. Immun.* **73**, 6026–6038.
16. Yoo, J. Y., Kim, H. C., Zhu, W., Kim, S. M., Sabet, M., Handfield, M., Hillman, J., Progulske-Fox, A., and Lee, S. W. (2007) Identification of *Tannerella forsythia* antigens specifically expressed in patients with periodontal disease. *FEMS Microbiol. Lett.* **275**, 344–352.
17. Handfield, M., and Hillman, J. D. (2006) In vivo induced antigen technology (IVIAT) and change mediated antigen technology (CMAT). *Infect. Disord. Drug Targets*. **6**, 327–334.
18. Handfield, M., Brady, L. J., Progulske-Fox, A., and Hillman, J. D. (2000) IVIAT: a novel method to identify microbial genes expressed specifically during human infections. *Trends Microbiol.* **8**, 336–339.
19. Handfield, M., Seifert, T., and Hillman, J. D. (2003) In vivo expression of bacterial genes during human infections. *Methods Mol. Med.* **71**, 225–242.

Chapter 14

Oral Bacterial Genome Sequencing Using the High-Throughput Roche Genome Sequencer FLX System

Nicholas C.K. Heng and Jo-Ann L. Stanton

Abstract

For over 30 years, the chain termination method of DNA sequencing (commonly known as Sanger sequencing) has been the mainstay of any DNA sequencing project. In the past, whole-genome sequencing employing exclusively Sanger chemistry has been a labor-intensive and costly exercise and an option unfeasible for the average research group. However, within the last 4 years, the introduction of three high-throughput sequencing technologies (454, SOLiD, and Illumina) has revolutionized genomics by facilitating unprecedented levels (up to gigabasepairs) of reliable DNA sequence output in a relatively short time frame and at a much lower cost per sequenced basepair. Here, we provide laboratory and bioinformatic protocols that will allow the average research group to undertake high-throughput sequencing of oral bacterial genomes using the Roche Genome Sequencer FLX System which employs 454 pyrosequencing technology.

Key words: Oral bacterial genome sequencing, *Streptococcus*, genomic DNA purification, Roche Genome Sequencer FLX System, 454 shotgun pyrosequencing, bioinformatics, Linux, Newbler genome assembler, GeneMark.hmm gene prediction software.

1. Introduction

Since its introduction over 30 years ago, the chain termination technique of DNA sequencing (also commonly called the Sanger sequencing method (1)) has been the mainstay of any project involving characterization of genetic material at the nucleotide level. Initially utilizing radioactively labeled synthesis-terminating dideoxynucleotides in combination with DNA polymerase and X-ray films, numerous advances including (a) the replacement of radiolabels with fluorophores, (b) the use of thermostable

DNA polymerases, (c) improved chemistries, and (d) the development of more sophisticated fluorescence detection instrumentation have allowed the Sanger method to generate >900 basepairs (bp) of reliable nucleotide sequence data (2). Moreover, miniaturization has allowed 96 sequencing reactions to be conducted simultaneously, yielding 96 kbp of DNA sequence in a single run (2).

The human oral cavity is home to >700 microbial species (3), collectively termed the oral microbiota. Although many bacterial species are culturable in the laboratory and can be studied to great detail, much is still unknown of the functions and activities of most, if not all, oral species. One of the most direct steps to redress the paucity of information regarding the activities of oral microbes is to perform whole-genome sequencing on important species. The Human Oral Microbiome Database (<http://www.homd.org>) is a recently established resource featuring completely sequenced oral bacterial genomes. In an ideal world, research groups would have access to all the genomic secrets of their favorite organism in order to facilitate downstream studies such as transcriptomics. However, whole-genome sequencing, especially those involving purely Sanger-based sequencing, is labor intensive, time consuming, and expensive. For example, a 96-reaction run yielding less than 100 kbp would cost the average laboratory group between \$400 and \$600 (a few dollars per sequence read). Furthermore, this cost excludes the cost of cloning DNA fragments (in *Escherichia coli*) and/or synthesis of polymerase chain reaction (PCR) primers.

Less than 5 years ago, the first of several next-generation DNA sequencing technologies called “pyrosequencing” (also known as “454 sequencing”) was introduced (4). Pyrosequencing consists of a high-throughput highly miniaturized “PCR on a droplet” system which relies on the release of pyrophosphate during DNA polymerization, ultimately leading to the production of photons (light), which are subsequently detected. The prototype 454 sequencing system (called the GS20) allowed >300,000 individual sequencing reactions to be analyzed in a single run (4). At the time of writing, the third-generation 454 pyrosequencer, called the Roche Genome Sequencer FLX (GS-FLX) Titanium System, is capable of analyzing over a million individual pyrosequencing reactions, yielding an unprecedented >500 megabasepairs (Mbp) of reliable sequence data with an average read length of 400 bp. As the nature of pyrosequencing is indiscriminate, cloning bias is eliminated as there is no need for prior cloning of genes. Although the competing next-generation sequencing technologies, namely the Applied Biosystems SOLiD System (5) and the Illumina (Solexa) Genome Analyzer (6), both generate far more sequence data (over 15 gigabasepairs) per run, their read lengths are short (<100 bp). Indeed, the GS-FLX appears to

be the preferred system when performing de novo whole-genome sequencing, sampling microbial diversity and metagenomics (3, 7–10). Regardless of the technology employed, the cost of next-generation DNA sequencing is relatively miniscule (a fraction of a cent) compared to conventional DNA sequencing by the Sanger method. Despite this, the Sanger method will still be required for the gap closure phase (*see* below) of any genome sequencing project.

Our research group has recently commenced whole-genome sequencing of strains of a ubiquitous and important oral *Streptococcus* species. In this chapter, we present laboratory and bioinformatic protocols that will allow the average research group to begin undertaking high-throughput sequencing of oral bacterial genomes using the second-generation Genome Sequencer FLX Pyrosequencer.

2. Materials (*see* Note 1)

2.1. Growth and Storage Media for Oral Bacteria

1. Liquid media for growing oral bacteria (e.g., streptococci): Bacto™ Brain Heart Infusion broth base (BHI) 37 g (per liter). BHI medium consists of BHI containing 0.5% yeast extract (5 g/L). Sterilize by autoclaving at 121°C for 20 min (*see* Note 2).
2. Solid media for subculturing oral bacteria (applicable to most, if not all, culturable oral species): BHI blood agar consisting of BHI broth base (37 g/L), 1.5% agar (15 g/L), and 5% defibrinated horse, human, or sheep blood. Sterilize the BHI agar first (121°C for 20 min) and cool to 50–55°C before adding the blood and dispense immediately (ca. 20 mL volumes) into petri dishes.
3. Anaerobic glove box (e.g., DW Scientific Modular Atmosphere Controlled System) or GasPak™ EZ Anaerobe Container System (Becton Dickinson & Co.) for growing anaerobic oral bacteria.
4. Disposable 1 mL plastic cuvettes (1 cm path length).
5. Spectrophotometer for measuring optical densities (e.g., Jenway 6300).
6. Sterilized (autoclaved) 100% glycerol (for stock cultures).
7. 1.5 mL cryovials or equivalent (for long-term storage of stock cultures).

2.2. Purification of Genomic DNA from Oral Bacteria

1. 15 mL Falcon centrifuge tubes.
2. Sterile phosphate-buffered saline (PBS; 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.4). Sterilize

either by membrane filtration (0.22 μm pore size) or by autoclaving (121°C for 20 min) (*see Note 3*).

3. *Optional* DNeasy blood and tissue genomic DNA purification kit (Qiagen GmbH, Hilden, Germany) (*see Note 4*).
4. *Optional* DNeasy enzymatic lysis buffer: 20 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1.0% Triton X-100. Sterilize by autoclaving (20 min, 121°C). Add lysozyme (Sigma-Aldrich, St. Louis, MO) to 20 mg/mL immediately before use.
5. *Optional* PureLink genomic DNA purification kit (Invitrogen, Carlsbad, CA) (*see Note 4*).
6. *Optional* PureLink genomic digestion buffer: 25 mM Tris-HCl, 2.5 mM EDTA, 1.2% Triton X-100. Sterilize by autoclaving (121°C, 20 min). Add lysozyme to 20 mg/mL prior to use.
7. Refrigerated microcentrifuge (e.g., Eppendorf model 5415R).
8. Microvolume spectrophotometer [e.g., NanoDrop (Thermo Scientific, Wilmington, DE) or NanoVue (GE Healthcare Life Sciences)] for measuring DNA concentrations.

2.3. GS-FLX Sequencing Library Construction

1. GS DNA Library Preparation Kit (Roche, Penzberg, Germany) (*see Note 5*).
2. RNA 6000 Pico LabChip[®] (Agilent Technologies, Santa Clara, CA).
3. DNA 7500 LabChip[®] (Agilent Technologies).
4. MinElute PCR purification kit (Qiagen).
5. AMPure 60 kit (Agencourt Bioscience, Beverly, MA).
6. RiboGreen RNA quantitation kit (Invitrogen).
7. 10 N NaOH.
8. Glacial acetic acid.

2.4. GS-FLX High-Throughput Sequencing

1. GS Emulsion PCR (emPCR) kit I (Roche) (*see Note 5*).
2. GS LR70 sequencing kit (Roche).
3. GS LR25 sequencing kit (Roche).
4. GS PicoTiter Plate kit (70 $\times$ 75) (Roche).
5. GS PicoTiter Plate kit (25 $\times$ 75) (Roche).
6. TissueLyser II tissue disruptor (Qiagen).
7. Platinum[®] High Fidelity *Taq* DNA polymerase (Invitrogen).
8. Eppendorf Twin.tec 96-well plates and seals.

9. Swin-Lok Filters (Whatman, Kent, United Kingdom).
10. Genome Sequencer FLX instrument (Roche).

2.5. Post-sequencing Bioinformatics

1. A computer workstation with the following *minimum* hardware requirements: (i) a dual-core Intel- or AMD-based central processing unit (CPU) running at 2+ gigahertz (GHz), (ii) 2 gigabytes (GB) of random access memory (RAM), and (iii) 160 GB hard disk drive (*see* **Note 6**).
2. 32-bit or 64-bit Linux operating system (*see* **Notes 7 and 8**).
3. Roche GS-FLX System Off-Instrument Software version 2.0.0.20 (or the older 1.0.3.24) (*see* **Note 9**).
4. Access to the Internet and a World Wide Web browser (e.g., Internet Explorer and Firefox).

3. Methods

3.1. Growth of Oral Bacteria

The following steps apply to oral inhabitants of the genus *Streptococcus* and related species. Other more fastidious oral bacteria, e.g., *Porphyromonas gingivalis*, will require additional growth factors such as hemin and menadione.

1. Grow the bacterial strain of interest on BHI blood agar. Ensure that well-isolated colonies are obtained.
2. Transfer a single bacterial colony into a 10 mL BHI (or BHY) broth. Incubate overnight (16–18 h) at 37°C under anaerobic conditions. Prewarm and prereduc a 15 mL BHI/BHY broth under the same conditions.
3. The next day, inoculate the 15 mL BHI/BHY broth with 150 µL (1% inoculum) of overnight culture. Incubate the broth culture anaerobically at 37°C until an optical density at 600 nm (OD₆₀₀) of 0.5 is reached (*see* **Note 10**).
4. Remove 850 µL aliquot of cells and place in a 1.5 mL cryovial. Add 150 µL of sterile 100% glycerol (final concentration of 15%) and mix by vortexing. Store the cryovial at –80°C. This is now the stock culture of the genome strain. The storage of multiple cryovials of the strain is highly recommended.
5. The remainder of the culture can be carried over to the genomic DNA purification step.

3.2. Purification of Genomic DNA

The following protocol can be used for both the DNeasy and PureLink DNA purification kits.

1. Harvest the cells from 10 mL of culture by centrifugation (8,000*g* for 10 min) in 15 mL Falcon centrifuge tubes (*see Note 11*).
2. Wash the cells twice in sterile PBS by vortexing and centrifuging as above.
3. Resuspend the cell pellet in 500 μ L of enzymatic (DNeasy) or digestion (PureLink) buffer, each containing 20 mg/mL lysozyme. Divide the cell suspension equally into two microfuge tubes and incubate at 37°C for 1 h with gentle inversion of the tubes every 15 min.
4. Add the lysis buffer (usually containing proteinase K to degrade the proteins) as per the manufacturer's instructions (*see Note 12*). The lysate will generally be clear and become quite viscous.
5. Proceed with the spin column sections of the purification protocol as per the manufacturer's instructions. Note that two lots of lysates will initially be spun through a single purification column.
6. Elute the genomic DNA in 100 μ L of the elution buffer provided by the manufacturer.
7. Quantify the amount of genomic DNA using a microvolume spectrophotometer such as a NanoDrop ND-1000. Ideally, the purity of the DNA (A_{260}/A_{280} ratio) should be ~ 1.8 .
8. Although optional, it would be prudent to perform PCR experiments to verify that the genomic DNA preparation is indeed correct. This can include Sanger-based sequencing of the 16S rDNA gene and/or verifying the presence of known genes in the strain. Moreover, these experiments will also detect any impurities, e.g., inhibitors of PCR, that may be present in the DNA preparations.
9. Subject 3–5 μ g (diluted to a concentration of 50 ng/ μ L) of purified genomic DNA for library construction and subsequent high-throughput sequencing using the GS-FLX system (*see below*).

3.3. GS-FLX Sequencing Library Construction

Due to the expenses incurred for consumables and the requirements of specialized equipment, it is highly recommended that any average laboratory undertaking genome sequencing projects utilize the services of a dedicated sequencing facility to carry out the GS-FLX library construction and GS-FLX sequencing procedures. In the following sections, we will describe the protocols (and the principles behind them) that are carried out within the Otago High-Throughput DNA Sequencing Facility (*see Note 13*).

Before commencing library construction it is important to ensure that the genomic DNA to be sequenced is free of contaminants ($OD_{260/280}$ should be as close to 1.8 as possible) and is not degraded (*see Note 14*).

1. Between 3 and 5 μ g of genomic DNA is fragmented by nebulization with nitrogen gas at 45 psi for 1 min. This breaks the genome into random fragments less than ca. 800 basepairs (bp).
2. Fragments <300 bp are removed using AMPure beads according to the supplier's instructions.
3. The DNA is then checked using a DNA 7500 LabChip[®] to ensure the sample consists of DNA fragments between 300 and 800 bp in size (*see Note 15*).
4. The DNA fragments are end-repaired (blunt-ended) using T4 DNA polymerase and T4 polynucleotide kinase followed by blunt-end ligation of the GS-FLX sequencing adaptors to the DNA. The sequencing process requires a different adaptor which is ligated to each end of the DNA fragment. These adaptors are referred to as the "A Adaptor" and "B Adaptor."
5. Fragments with the required conformation (i.e., A Adaptor + DNA Fragment + B Adaptor) are selected using a streptavidin/biotin magnetic bead system. One strand of the B Adaptor is biotinylated. DNA fragments with a B Adaptor at one or both ends bind irreversibly to the streptavidin-coated magnetic beads. Molecules with either (a) no adaptors or (b) only A Adaptors are removed by washing steps.
6. Fragments with an A Adaptor and B Adaptor at each end are captured by melting the DNA tethered to the streptavidin beads with 0.125 M NaOH. This releases the single-stranded DNA sequencing library. DNA molecules with B Adaptors at both ends remain tethered to the beads as both strands of the molecule are biotinylated.
7. The NaOH, in which the single-stranded DNA sequencing library is suspended, is neutralized using a Qiagen MinElute spin column in which the pH of the first PBI column wash is adjusted using acetic acid.
8. A 1 μ L aliquot of the purified single-stranded DNA sequencing library is then checked on a RNA Pico 6000 LabChip[®] to ascertain the average fragment length of the DNA sequencing library.
9. The concentration of the library is measured fluorometrically using RiboGreen. The number of molecules in the library is determined using these two parameters in the equation

$$\text{Molecules}/\mu\text{L} = \frac{\text{Sample} \times \text{Avogadro's constant}}{(328.3 \times 10^9) \times (\text{fragment length})}$$

where Sample = the concentration of the Library in ng/ μL ; Avogadro's constant = 6.022×10^{23} molecules/mol; 328.3×10^9 is the average molecular weight of a nucleotide in g/mol; and fragment length = the number of nucleotides in the average-sized fragment in the library.

10. Once prepared, libraries are diluted to 1×10^8 molecules/ μL , aliquoted, and stored at -20°C until use (*see* **Note 16**).

3.4. GS-FLX High-Throughput Sequencing

Preparation of libraries for sequencing is preformed in two stages. First, sequencing libraries are used in emulsion PCR (emPCR) to capture, or chemically clone, each molecule onto a substrate bead (referred to as a "capture bead"). Second, enriched DNA-containing capture beads are loaded onto a PicoTiter Plate and used on a Genome Sequencer FLX instrument for the pyrophosphate sequencing phase.

3.4.1. Emulsion PCR (emPCR)

1. An aliquot of DNA sequencing library is added to capture beads. These capture beads are coated with oligonucleotides complementary to the B Adaptor.
2. Capture beads with the annealed library are added to emulsion oil along with the components for PCR, these being Platinum High Fidelity *Taq* DNA polymerase, *Taq* reaction buffer, nucleotides, and an oligonucleotide primer complementary to the A Adaptor.
3. The emulsion oil, capture beads, and PCR components are mixed vigorously using a TissueLyser at a frequency of 15 Hz for 5 min. This disperses the capture beads throughout the oil in small water droplets approximately 50–100 μm in diameter. These are referred to as "microreactors" as these droplets also contain the reaction components for PCR.
4. The emulsion is then distributed into 96-well Eppendorf Twin.tec plates and thermocycled once at 94°C for 4 min, followed by 40 cycles of 94°C for 30 s, 58°C for 60 s, and 68°C for 90 s. Finally, 13 cycles of 94°C for 30 s and 58°C for 6 min complete the thermocycling program. This process amplifies each DNA molecule so that each capture bead is coated in thousands of copies of a single DNA sequence. This process is necessary so that the signal

generated during the sequencing process is sufficient for the GS-FLX instrument to detect.

5. Following amplification the emulsions are broken to remove the oil. The emPCR sequencing beads are collected in a single 10 mL syringe.
6. A Swin-Lok filter is attached to the syringe and the capture beads washed three times with approximately 9 mL of isopropanol (propan-2-ol).
7. The beads are then washed three times with a proprietary enhancing fluid and then removed from the Swin-Lok filter by rapidly drawing air back through the membrane.
8. The washed beads are then enriched for those carrying DNA. Magnetic beads coated with a complementary oligonucleotide to the A adaptor are annealed to the capture beads. A magnet is used to hold the capture beads to which magnetic beads have annealed and the capture beads with no attached DNA are washed away. Addition of 0.125 M NaOH displaces the bound capture beads from the magnet and, at the same time, denatures the DNA on the capture beads into single-stranded DNA.
9. The ratio of DNA sequencing library to capture beads used in each emPCR must lead to each bead capturing only one DNA molecule. If capture beads contain more than one DNA molecule the sequence from the bead cannot be resolved. To obtain the correct ratio, capture beads must be in excess of the number of DNA molecules in the reaction such that, if a capture bead encounters a DNA molecule, it will only do so once according to a Poisson distribution. The ratio of single-stranded DNA sequencing library added to capture beads is determined by titration. The titration is performed using four DNA-to-capture bead ratios: (i) 0.5 molecules/bead, (ii) 2 molecules/bead, (iii) 4 molecules/bead, and (iv) 16 molecules/bead. These emulsions are broken, enriched, and sequenced using a small LR25 format sequencing kit to determine the optimal DNA-to-capture bead ratio. This should be the amount of DNA that gives approximately 3,000 keypass wells (*see below*).
10. A sequencing primer complementary to 15 nucleotides of the A adaptor is then annealed to the DNA on the capture beads. The capture beads are now ready for sequencing.

3.4.2. Pyrophosphate Sequencing

Pyrophosphate sequencing is carried out using two components: (a) a PicoTiter Plate and (b) the Genome Sequencer FLX instrument. The Genome Sequencer FLX provides the sequencing reaction with reagent washes in a cycled series and captures light

output from the PicoTiter Plate as an ordered number of images, each of which corresponds to the specific nucleotide incorporated into the DNA strand at a given position along the strand. The PicoTiter Plate holds each DNA capture bead in a defined position throughout the sequencing process so that images collected from the plate can be aligned and the sequence, represented by light output from each bead, can be read.

1. Enriched capture beads in solution are loaded onto a PicoTiter Plate. The PicoTiter Plate consists of bundles of fused optic fibers oriented to run perpendicularly through the plane of the plate. One side of the plate is etched so that the center of each fiber is removed faster than the edges fused to neighboring fibers. This forms a well with a diameter of 40 μm . The other side of the plate is polished so that the bottom of each well is an optically clear window. The PicoTiter Plate is sealed into a bead deposition device and, once the solution of capture beads is added, incubated flat on a bench for 10 min to allow gravity to settle the beads into the wells. Theoretically, only one bead can fit into each well due to its size.
2. Two additional layers of beads are then added to the plate and centrifuged into place. These two bead layers perform two functions: (i) they supply each well with the enzymes for pyrophosphate sequencing and (ii) they fill in all available space around the capture bead, i.e., packing it into place so it cannot move from the well. This packing of each capture bead is imperative as the reader will notice below.
3. The pyrophosphate reaction uses the consecutive actions of three enzymes: polymerase, sulfuryase, and luciferase. The polymerase incorporates nucleotides into an extending complementary copy of DNA tethered to the capture bead starting from a sequencing primer annealed to the A Adaptor (*see* **Section 3.4.1**). Each time a nucleotide is incorporated, inorganic phosphate is released which the sulfuryase uses to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The luciferase in turn uses the ATP to convert its substrate, luciferin, and release a photon of light. This photon is then detected by a sophisticated charge-coupled device (CCD) camera that forms an integral part of the Genome Sequencer FLX instrument.
4. The DNA sequence is determined by supplying only one nucleotide at a time to the reaction, i.e., the sequential cycling of nucleotides. So, in the first round, only thymine (T) is supplied. Where T is the next base in the DNA molecule, it is incorporated by the polymerase, and the well in which that particular bead sits emits light (photon). If T is not the next base, the well remains dark,

i.e., no polymerase–sulfurase–luciferase reaction cascade has occurred. T is purged from the system and the next base, for instance adenine (A), is supplied. As with T, where A is the next base, the well will light up. This is repeated for cytosine and guanine.

5. The cycle of nucleotide washes is then repeated up to 200 times. Aligning the images and noting when each well produces light with the corresponding base reveals the sequence read at that position. This process can produce sequence reads of up to 300 bp in length.
6. The intensity of light generated from each well is proportional to the amount of phosphate released during nucleotide incorporation (*see Note 17*). There are a number of factors that influence the amount of phosphate release and, thus, the light intensity from each well. These factors include the efficiency of emPCR of a particular sequence or whether the nucleotide is incorporated only once per wash or multiple times, as is the case in a homopolymer sequence (i.e., a run of more than one of the same nucleotide). This means that the baseline light output from each well must be calibrated. To achieve this, the first four bases sequenced for all reads is referred to as the “key sequence” and it represents the last four bases of the A Adaptor. This consists of the sequence “TCAG” and it sets the baseline light output for a single nucleotide incorporation for each well. When more than one nucleotide is incorporated during a particular nucleotide wash cycle, the light intensity from the well will be multiples of the baseline signal generated by that single nucleotide. For example, the light output from the sequence AA will be twice that for a single A incorporation (*see Note 17*). This relationship holds true for up to approximately eight incorporations of the same nucleotide. However, interpreting data for homopolymer sequences >8 bases requires caution.
7. Finally, all of the data from each PicoTiter Plate is subjected to five data quality filters. These filters remove (a) spurious signal potentially arising from dust particles, (b) sequences arising from wells in which two or more sequences are present, (c) sequences lacking a key sequence (keypass filter), (d) sequences consisting of the adaptors only, and (e) reads shorter than 80 bp. Sequences deemed to be of low quality are also removed from the final data set. Under optimal conditions, the final sequence output from a full PicoTiter Plate is approximately 420,000 sequence reads or >100 million bases of usable sequence data (*see Note 18*).

3.5. Post-sequencing Bioinformatics

3.5.1. Installing and Operating the GS-FLX Software Package

This section describes the installation and operation of the GS-FLX Off-Instrument software on a Linux-based workstation. The software *will not* work natively in a Microsoft Windows-based PC or on an Apple Macintosh computer. The commands and modifications provided here have been tested successfully on various Linux platforms (*see Note 8*). However, if difficulties are encountered, seek assistance from personnel knowledgeable in the use of the Linux operating system.

1. Install the Linux operating system (32-bit or 64-bit) on the designated bioinformatics workstation as appropriate for the computer's specifications. This is generally a very straightforward, menu-driven process.
2. Install the appropriate version (32-bit or 64-bit) of the Genome Sequencer FLX Off-Instrument Software. First, copy the GS-FLX software folder (called offInstrument-Apps_2.0.00.20; -2.0.00.20-64 for the 64-bit version) to the hard disk. Using the application called Terminal, change the active directory to the GS-FLX software folder.
3. Type the `./INSTALL` command. This will provide you with options as to what packages can be installed. Select "Off-Instrument Apps."
4. Provide the installation program with a suitable folder name, e.g., `/opt/454`. This is the folder in which the GS-FLX software files will be installed. Make note of the folder name as it is case sensitive.
5. The installation program will recommend that the PATH be modified in the `.bashrc` file to include the specified GS-FLX folder. The `.bashrc` file is a hidden login file that provides the operating system with options including PATH (names of folders where the operating system will look for files, commands, etc.).
6. Open the `.bashrc` file using the command `"gedit .bashrc"` (*see Note 19*).
7. Immediately under the heading `"# User specific aliases and functions,"` type `"export PATH=$PATH:{GS-FLX folder name}/bin."` Ensure that `/bin` is included as it is where all the GS-FLX binary files are located. For example, if the GS-FLX software has been installed in `/opt/454`, then type `"export PATH=$PATH:/opt/454/bin."` This will append `/opt/454/bin` to the list of folders in PATH. Save the `.bashrc` file, exit Terminal, and re-login to the operating system.

8. In order to run components of the GS-FLX software, simply type the name of the run-script in Terminal. For the purposes of this chapter, type “gsAssembler” to run the GS Assembler (Newbler) de novo DNA assembly program (*see* **Notes 20** and **21**).
9. The GS Assembler program will request that the user specify a directory for the genome sequence assembly output files.
10. Locate the directory in which the filtered, finalized GS-FLX sequence data (obtained in Step 7 of **Section 3.4.2**) has been deposited. The filename usually consists of a code and a SFF suffix, e.g., FKUBIG01.SFF. Add the relevant SFF file to the project.
11. Specify the parameters for the sequence assembly. Among the default parameters are 40 nucleotide minimum overlap, 90% identity minimum, 100 bp to qualify as a “contig,” 500 bp for large contigs (*see* **Note 22**). It is recommended that different parameters are tested as they will yield variable results.
12. Start the assembly process (*see* **Note 23**).
13. The overall assembly statistics are found in the 454NewblerMetrics.txt file. This will provide important information such as the number of sequence reads aligned, the overall error rate of the assembly, the largest contig obtained, and average contig length.
14. The contigs generated by the GS Assembler program will be saved in the file called 454AllContigs.fna. Similarly, large (>500 bp) contigs will be saved under the filename 454LargeContigs.fna. These files are actually very large text files that can be opened easily by any word processing software, e.g., Microsoft Word.
15. Each contig will be listed in FASTA format with a header, for example, “>contig00001 length = 32654 numreads = 2924,” followed by the associated nucleotide sequence (*see* **Note 24**).

3.5.2. Analyzing the Contigs Generated by the GS Assembler Software

Any genome sequence assembly, regardless of the level of sequence coverage, will always generate more than one contig. This is because the GS Assembler software utilizes an algorithm which aligns sequences according to a consensus overlap scheme. The total number of contigs obtained will depend on the assembly parameters specified by the user, with 100% identity between overlaps (i.e., the most stringent condition) invariably resulting in the highest number of contigs.

Among the known genetic elements that will contribute to the generation of multiple contigs are the repetitive DNA regions:

(a) ribosomal RNA (rRNA) operons, (b) mobile genetic cassettes such as insertion sequences (IS) and transposons (Tn), (c) untranslated (non-coding) repeat regions, and (d) genes that specify proteins with repeated amino acid motifs (*see* **Note 25**).

Our genome sequencing pipeline comprises the following stages:

- (a) High-throughput sequencing
 - (b) Contig sorting into chromosomal and extrachromosomal elements (if applicable)
 - (c) Determining the chromosomal contig order
 - (c) Gene prediction and detection for each contig
 - (d) Annotation of detected open reading frames (ORFs)
 - (e) Gap closure procedures (*see* below) and finalization of genome sequence
1. Contig sorting is carried out primarily using the BLASTN and BLASTX algorithms (**11**) (*see* **Note 26**). If there are no extrachromosomal elements (plasmids or megaplasms) present in the genome strain, and if there is a related reference genome sequence(s), then the order of the contigs can be deduced based on the relative coordinates of the reference. If your genome strain harbors extrachromosomal elements, the location of contigs can be determined by PCR, especially if a plasmid-free derivative is available (**Fig. 14.1**).
 2. Gene prediction can be performed using Web-based tools such as GeneMark.hmm (**12**) and FGENESB (<http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfindb>) (*see* **Note 27**). Another popular gene-hunting algorithm, GLIMMER 3.0 (**13**), can also be installed as a stand-alone (i.e., Web-free) program on the bioinformatics workstation. However, programs such as GLIMMER do not utilize a graphical user interface and also require input of commands (with parameters). We highly recommend the use of the GeneMark.hmm server (<http://exon.gatech.edu/GeneMark/>).
 3. Annotation of ORFs detected by GeneMark.hmm, FGENESB, or GLIMMER can be performed either manually (in combination with the BLASTP protein homology algorithm) or using Web-based servers such as RAST (<http://rast.nmpdr.org/>) and GenDB (**15**). However, such annotation servers are more effective once the complete genome sequence is available (*see* **Note 28**).

3.6. Gap Closure Procedures

For any genome sequencing project, the gap closure phase, i.e., the generation and incorporation of bridging sequences between adjacent contigs, is inevitable. To date, even the most straightforward GS-FLX-based genome project, i.e., that of the

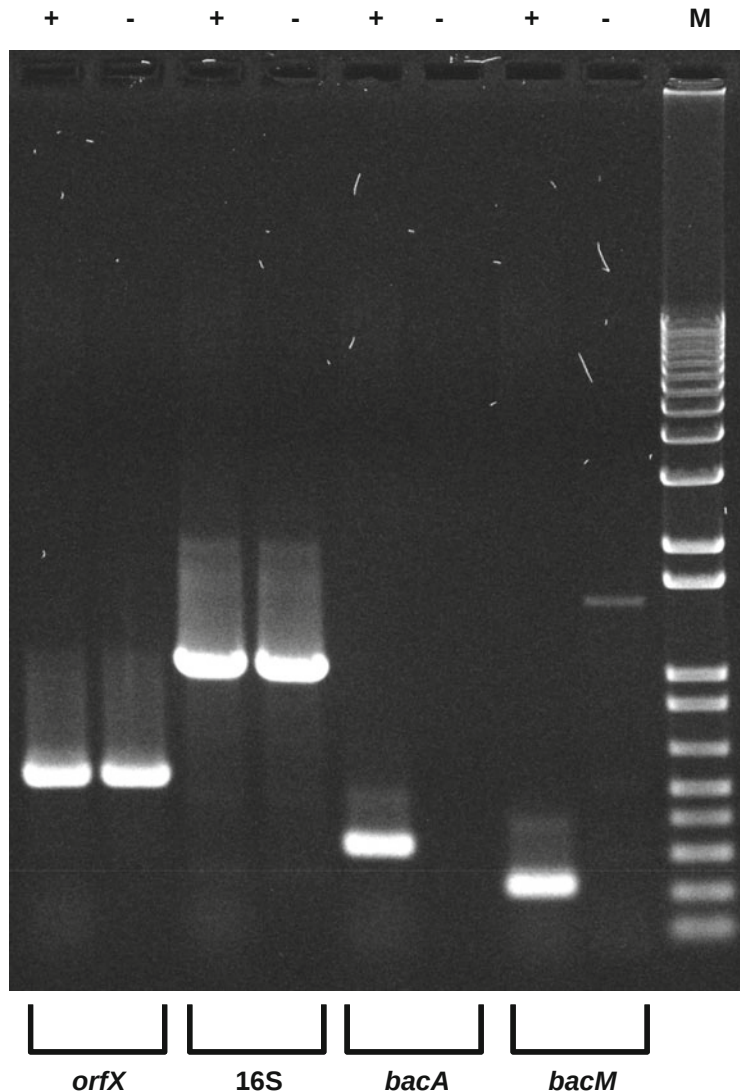


Fig. 14.1. Distinguishing chromosomal from megaplasmid contigs using PCR. Genomic DNA templates were purified from the wild-type (+) *Streptococcus* strain and its megaplasmid-negative derivative (-). An open reading frame (*orfX*), which encodes a large (>100 kDa) putative cell surface protein, was tested by PCR along with one known chromosomal locus (16S rDNA) and two megaplasmid-associated genes (*bacA* and *bacM*). The results indicate that *orfX* is located on the chromosome. Lane M contains the 1 kb+ DNA size marker (Invitrogen).

Gram-positive pathogen *Corynebacterium kroppenstedtii*, has required gap closure (6).

Gap closure involves the following steps:

1. Design of suitable oligonucleotide primers for PCR (*see Note 29*).

2. Amplification of inter-contig gap-spanning regions using high-fidelity PCR (*see* **Note 30**).
3. Sequencing of amplicons and incorporation of the new sequences into the existing genome sequence data set(s) (*see* **Note 29**).

Once the genome sequence has been finalized, it can be formatted and deposited into the GenBank DNA sequence database using the stand-alone Sequin (version 10.0 at the time of writing) DNA sequence submission software. Sequin is available for free download from <http://www.ncbi.nlm.nih.gov/Sequin/>.

4. Notes

1. All media and chemicals were purchased from Becton Dickinson & Co. (formerly Difco; Sparks, MD) and Sigma-Aldrich (St. Louis, MO), respectively.
2. This liquid media can also be used for culturing gram-negative bacteria, in particular the periodontopathogenic species (e.g., *Porphyromonas gingivalis*) but supplementation with vitamin K (menadione; 1 g/L) and hemin (1 g/L) is essential.
3. This is simply a wash buffer to remove most of the growth media components during harvesting of the bacterial cells. Some suppliers (e.g., Invitrogen) do provide PBS with different compositions (e.g., potassium phosphate) or pH values but any of these are also suitable. Do not use water to wash cells as some species may lyse as a result of osmotic shock.
4. The DNeasy and PureLink genomic DNA purification kits have been used successfully in our laboratory. However, the manufacturers of both kits do not provide the enzymatic/digestion (lysis) buffer and the end-user will have to prepare this beforehand.
5. If a service provider is approached to undertake GS-FLX sequencing, it is advisable that library construction, emPCR, and sequencing are all performed by the provider. They have the required materials, equipment, and expertise. Generally, the kits listed in **Sections 2.3** and **2.4** are supplied in relatively large quantities, thus making one-off applications proportionally more expensive. Specialist (and expensive) equipment not readily available in the average laboratory is also required for the process.

6. Our primary bioinformatics workstation consists of a 3-GHz quad-core Intel-based system with 8 GB of RAM and a 500 GB hard disk drive. Systems with much more modest specifications, e.g., a 2.2-GHz Intel Celeron CPU-based laptop computer with 384 MB of RAM and a 30 GB hard disk, have been on trial and can be used as long as the minimum hardware requirements for the Linux operating system are satisfied.
7. If your bioinformatics workstation has less than 4 GB of RAM installed, the 32-bit Linux version is recommended.
8. Although Roche recommends Red Hat Enterprise Linux (RHEL) version 4 or higher for their GS-FLX software suite, there is an annual subscription required. Our laboratory bioinformatics workstation runs the 64-bit version of CentOS v5.4, a free RHEL-like distribution. The GS-FLX analysis software has also been successfully installed and tested on the following 32-bit flavors of Linux: Ubuntu 9.04 (and its derivatives such as Linux Mint 9), Fedora versions 10 and 11, Mandriva 2010 (Beta version), and openSUSE 11.2 (Release Candidate). Some of the intricacies of installing the GS-FLX software are described in **Section 3.5**.
9. We recommend the latest version (2.0.0.20 at time of writing) as the installation procedure is far simpler than that of version 1.0.3.24, with which our preliminary *Streptococcus* genome assemblies were conducted. If you are upgrading from version 1 to version 2, be aware that even with the same data set, the number of contigs may increase – this is due to the more aggressive algorithm employed by version 2.
10. Mid-logarithmic growth phase ($OD_{600} = 0.5$) cells are preferred over stationary phase cells as (a) actively growing cells are easier to lyse and (b) the genomic DNA will be of higher quality as it is less likely to be degraded by nucleases released during stationary phase.
11. Although the manufacturers of both DNeasy and PureLink kits recommend processing cells harvested from a 5 mL bacterial culture, this protocol doubles the number of cells for a single spin column so that a higher yield of genomic DNA (5–10 μ g) is obtained. The DNA-binding capacity of the DNeasy and PureLink spin columns is 15–20 μ g.
12. Never vortex the lysate as shearing of high molecular weight can occur!! Always mix by gentle inversion (10 times).

13. For more detailed information regarding GS-FLX-based high-throughput sequencing, the reader is referred to the following web site: <http://www.genome-sequencing.com>.
14. Starting the library construction with degraded DNA leads to loss of sequence representation. The result will be regions of the genome sequenced at low coverage or, in extreme cases, missing sequence data altogether.
15. It is a good idea to take 1 μ L samples of the DNA sample (a) before nebulization, (b) after nebulization, and (c) post-AMPure size selection. All three samples should be run on a DNA 7500 LabChip in an Agilent Technologies 2100 BioAnalyzer instrument to monitor the fragmentation process.
16. Avoid all repeat freeze/thawing of libraries. Aliquots should only be used once after freezing. Repeated freeze/thaw leads to rapid degradation of the library and dramatically reduces library efficiency for emPCR. Retitrate libraries if they have been stored for a long period of time, even when stored at -20°C .
17. The graphical output of a pyrosequencing reaction, called a “flowgram,” is very different from that of a standard Sanger sequencing chromatogram in that it is represented by intensity peaks rather than a defined peak for a particular base. The reader is referred to the GS-FLX web site (<http://www.genome-sequencing.com>) for examples of flowgrams.
18. The recently released GS-FLX Titanium upgrade boasts >1 million sequence reads with longer (~ 400 bp) read lengths and 500 million bp of usable sequence data.
19. Depending on the flavor of Linux, the command “sudo gedit .bashrc” or “su gedit .bashrc” may be required. The “sudo” and “su” are super-user commands and these usually require a password which you would have specified during installation of the Linux operating system.
20. *Important Note:* In Linux distributions of the Debian lineage such as Ubuntu and its derivatives (Linux Mint, etc.), the “gsAssembler” command will generate a “Syntax error” message. In this instance, you will need to edit the gsAssembler run-script located in the application’s folder. For example, if the GS-FLX software is installed in the /opt/454 folder, then the gsAssembler run-script will be found in the /opt/454/apps/assembly/bin/folder. The first line in the run-script is “#!/bin/sh” which you must change to “#!/bin/bash”. This alteration tells the operating system to use the proper command shell (i.e., the Bourne-again shell) to interpret the run-script. The

“gsAssembler” command should now run the desired program. Note that this .sh to .bash change *must* be performed in the run-scripts of the other components of the GS-FLX software for them to work properly in Ubuntu Linux (*see Note 21*).

21. The other components of the GS-FLX software include (a) gsMapper for the mapping program, (b) gsAmplicon for the GS Amplicon Variant Analyzer (to detect SNPs, etc.), and (c) gsRunBrowser for the RunBrowser program which allows visualization of the GS-FLX run data. The gsMapper, gsAmplicon, and gsRunBrowser run-scripts are located in {GS-FLX folder}/454/apps/mapper/bin/, {GS-FLX folder}/454/apps/amplicons/bin/, and {GS-FLX folder}/454/apps/runBrowser/bin/, respectively.
22. The default parameters will tell the Assembler algorithm to accept sequence alignments that overlap by at least 40 bp and of those, at least 90% (i.e., >36 bp) are identical. Any aligned sequence read groups comprising <100 bp total length will be excluded as a “contiguous sequence” or “contig.” Furthermore, any contig of >500 bp will qualify as a “large” contig.
23. For a typical 2-Mbp bacterial genome, assembly times for the same data set can range from 3 min (default workstation featuring an Intel quad-core CPU) to over 45 min (laptop containing an Intel single-core Celeron CPU).
24. The numreads value indicates the number of sequence reads contributing to that particular contig. In order to calculate the sequence coverage of any contig, use the following formula: $(\text{numreads} \times \text{average read length}) / \text{contig length}$. So for our example, in which the average read length was 248, the sequence coverage would be $(2,924 \times 248) / 32,654 = \sim 22$ -fold. Also note that (a) the nucleotide sequence can comprise upper case and lower case characters, e.g., TAGCTGTGCTGAAAaTGCT, and (b) the lower case “a” (an “unsure” basecall) in the sequence above is part of a homopolymeric run of As, a known limitation of the GS-FLX system.
25. Most bacterial genomes will contain more than one rRNA operon and all rRNA operons, due to the GS Assembler consensus overlap algorithm, will always yield a single contig (6). Our *Streptococcus* genome strains each have six rRNA operons, a multitude of IS and Tn cassettes, several repetitive (non-coding) DNA elements, and at least one chromosomal gene (e.g., *orfX* in Fig. 14.1) encoding a large cell surface protein with repeated amino acid motifs.

26. We use the BLASTN (nucleotide vs. nucleotide) and BLASTX (nucleotide vs. translated DNA) homology detection algorithms to interrogate the GenBank DNA database for entries similar to the contig sequence of interest (the “query”). The BLASTN results will indicate a possible reference genome for your strain of interest. For example, many of the contigs from our *Streptococcus* genome strains exhibited high homology to regions of the completely sequenced genome of the dairy organism *Streptococcus thermophilus*, which subsequently served as the reference sequence. On the other hand, the BLASTX results will provide some idea of whether the query contig contains unique genes, especially if there is significant homology to a known protein but negligible similarity at the nucleotide level (i.e., a negative BLASTN result). Moreover, if your genome strain has extrachromosomal elements such as megaplasms, the BLASTX result may be useful in sorting chromosomal from non-chromosomal contigs.
27. Both GeneMark.hmm and FGENESB use gene prediction algorithms that have been “trained” on a variety of completely sequenced bacterial genomes. Always select the organism that is most related to your genome strain.
28. We currently annotate our individual genomic contigs manually using a tab-delimited table format, which we can convert to ASN (GenBank database) format when all ORFs have been annotated. The tbl2asn program provided by GenBank will facilitate the conversion process.
29. An important consideration is that PCR primers be designed such that their binding sites are at least 300 bp from the end of the contig, and that they bind to *unique* sequences. In our experience, examples of non-unique sequences include the 5′ and 3′ ends of IS elements located at the termini of contigs – we have found that their central portions cluster as different contigs due to internal variability. As a result of designing primers to these non-unique sequences, some gap-spanning sequences were incorrectly incorporated. Software packages such as the multi-platform Consed (16) are recommended for the gap closure phase. Consed contains Autofinish, a module that facilitates the design of suitable PCR primers, and also allows mixed assembly of DNA sequences originating from different sources, e.g., 454 and Sanger. Consed (currently version 19) is downloadable, upon receipt of an academic license from the developers (16), from <http://www.phrap.org/consed/consed.html>.

30. DNA polymerases such as Platinum High Fidelity *Taq* (Invitrogen), KOD (Novagen) and PrimeStar (Takara) are recommended for the gap closure phase. However, these high-fidelity enzymes can be fairly costly. As a rule of thumb, gap-spanning sequences estimated to be <800 bp in size can be amplified using the cheaper regular Hot-Start *Taq* DNA polymerase such as HotMaster *Taq* (5Prime, formerly Eppendorf) or FastStart (Roche).

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References

1. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
2. Morozova, O., and Marra, M. A. (2008) Applications of next-generation sequencing technologies in functional genomics. *Genomics* **92**, 255–264.
3. Keijser, B. J., Zaura, E., Huse, S. M., van der Vossen, J. M., Schuren, F. H., Montijn, R. C., ten Cate, J. M., and Crielaard, W. (2008) Pyrosequencing analysis of the oral microflora of healthy adults. *J. Dent. Res.* **87**, 1016–1020.
4. Margulies, M., Egholm, M., Altman, W. E., Attiya, S., Bader, J. S., Bemben, L. A., Berka, J., Braverman, M. S., Chen, Y. J., Chen, Z., Dewell, S. B., Du, L., Fierro, J. M., Gomes, X. V., Godwin, B. C., He, W., Helgesen, S., Ho, C. H., Irzyk, G. P., Jando, S. C., Alenquer, M. L., Jarvie, T. P., Jirage, K. B., Kim, J. B., Knight, J. R., Lanza, J. R., Leamon, J. H., Lefkowitz, S. M., Lei, M., Li, J., Lohman, K. L., Lu, H., Makhijani, V. B., McDade, K. E., McKenna, M. P., Myers, E. W., Nickerson, E., Nobile, J. R., Plant, R., Puc, B. P., Ronan, M. T., Roth, G. T., Sarkis, G. J., Simons, J. F., Simpson, J. W., Srinivasan, M., Tartaro, K. R., Tomasz, A., Vogt, K. A., Volkmer, G. A., Wang, S. H., Wang, Y., Weiner, M. P., Yu, P., Begley, R. F., and Rothberg, J. M. (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376–380.
5. Shendure, J., Porreca, G. J., Reppas, N. B., Lin, X., McCutcheon, J. P., Rosenbaum, A. M., Wang, M. D., Zhang, K., Mitra, R. D., and Church, G. M. (2005) Accurate multiplex polony sequencing of an evolved bacterial genome. *Science* **309**, 1728–1732.
6. Bennett, S. (2004) Solexa Ltd. *Pharmacogenomics* **5**, 433–438.
7. Tauch, A., Schneider, J., Szczepanowski, R., Tilker, A., Viehoveer, P., Gartemann, K. H., Arnold, W., Blom, J., Brinkrolf, K., Brune, I., Götter, S., Weisshaar, B., Goesmann, A., Dröge, M., and Pühler, A. (2008) Ultrafast pyrosequencing of *Corynebacterium kroppenstedtii* DSM44385 revealed insights into the physiology of a lipophilic corynebacterium that lacks mycolic acids. *J. Biotechnol.* **136**, 22–30.

8. Aury, J. M., Cruaud, C., Barbe, V., Rogier, O., Mangenot, S., Samson, G., Poulain, J., Anthouard, V., Scarpelli, C., Artiguenave, F., and Wincker, P. (2008) High quality draft sequences for prokaryotic genomes using a mix of new sequencing technologies. *BMC Genomics* **9**, 603.
9. Zhang, H., DiBaise, J. K., Zuccolo, A., Kudrna, D., Braidotti, M., Yu, Y., Parameswaran, P., Crowell, M. D., Wing, R., Rittmann, B. E., and Krajmalnik-Brown, R. (2009) Human gut microbiota in obesity and after gastric bypass. *Proc. Natl. Acad. Sci. USA* **106**, 2365–2370.
10. Wolcott, R. D., Gontcharova, V., Sun, Y., and Dowd, S. E. (2009) Evaluation of the bacterial diversity among and within individual venous leg ulcers using bacterial tag-encoded FLX and titanium amplicon pyrosequencing and metagenomic approaches. *BMC Microbiol.* **9**, 226.
11. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
12. Besemer, J., and Borodovsky, M. (2005) GeneMark: web software for gene finding in prokaryotes, eukaryotes and viruses. *Nucleic Acids Res.* **33**(Web Server Issue), W451–W454.
13. Delcher, A. L., Bratke, K. A., Powers, E. C., and Salzberg, S. L. (2007) Identifying bacterial genes and endosymbiont DNA with Glimmer. *Bioinformatics* **23**, 673–679.
14. Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. (2008) The RAST server: rapid annotations using subsystems technology. *BMC Genomics* **9**, 75.
15. Meyer, F., Goesmann, A., McHardy, A. C., Bartels, D., Bekel, T., Clausen, J., Kalinowski, J., Linke, B., Rupp, O., Giegerich, R., and Pühler, A. (2003) GenDB – an open source genome annotation system for prokaryote genomes. *Nucleic Acids Res.* **31**, 2187–2195.
16. Gordon, D., Abajian, C., and Green, P. (1998) Consed: a graphical tool for sequence finishing. *Genome Res.* **8**, 195–202.

Chapter 15

Use of a Yeast-Based Membrane Protein Expression Technology to Overexpress Drug Resistance Efflux Pumps

Erwin Lamping and Richard D. Cannon

Abstract

Azole antifungal drugs are used widely to treat people with oral fungal infections. Unfortunately, fungi can develop resistance to these drugs. This resistance can be due to the overexpression or mutation of cytochrome P450 14 α -lanosterol demethylase, also known as *ERG11* or *CYP51*, and/or the overexpression of membrane-located multidrug efflux pumps. We have developed a heterologous membrane protein expression system that can be used to study the structure and function of these proteins in the non-pathogenic, genetically stable, and versatile eukaryotic model organism, *Saccharomyces cerevisiae*. In this chapter we describe the techniques used to express the *Candida albicans* efflux pump Cdr1p in *S. cerevisiae*.

Key words: Antifungal drug resistance, ABC multidrug efflux pumps, azole drug target, *ERG11*, *CYP51*, membrane proteins, heterologous expression, *Saccharomyces cerevisiae*.

1. Introduction

It is estimated that the fungal kingdom comprises about 1.5 million species of which about 200 are associated with humans (1). Most of these are harmless to healthy individuals and form part of the commensal microflora of the oral cavity or the gastrointestinal tract. However, some commensal fungi such as *Candida albicans* are opportunistic pathogens and can cause both superficial mucosal infections and serious, life threatening, invasive fungal infections (IFI) in immunocompromised individuals (2).

Paradoxically, increased treatment options due to advancements in modern medicine, the rise of the AIDS epidemic, an

“ageing” population of affluent countries, and poor living conditions due to poverty have together led to a rising number of immunocompromised individuals that are susceptible to these opportunistic fungal infections. A number of *Candida* species, some *Cryptococcus neoformans* strains and *Aspergillus* species, with *Aspergillus fumigatus* being the most prevalent, are among the leading causes of IFI. Despite the threat that the increasing incidence of IFI poses, only a limited number of antifungal drugs are available to the clinician to treat patients with systemic fungal infections.

Azoles, such as the first-generation triazoles fluconazole or itraconazole or the second-generation triazoles voriconazole, posaconazole, or ravuconazole, are the most widely used class of antifungals to treat patients with IFI. The azole target is cytochrome P450 14 α -lanosterol demethylase, encoded by the *ERG11* gene (also known as *CYP51*), a key enzyme in ergosterol biosynthesis (3).

Ergosterol is an essential, amphiphilic, sterol component of fungal cell membranes, while mammalian cells possess cholesterol, and most plant species have sitosterol and stigmasterol. Exposure of fungal cells to inhibitory concentrations of azoles depletes their cell membranes of this essential component. Unfortunately, azoles are in most cases fungistatic rather than fungicidal. This allows fungal cells to survive long enough to develop resistance through adaptation and mutagenesis. Mechanisms of azole resistance in *C. albicans* include the mutation or the overexpression of the drug target, *ERG11*, with highest levels of resistance caused by the overexpression of multidrug efflux pumps such as CaCdr1p, CaCdr2p, or CaMdr1p (4, 5). These pumps are integral membrane proteins located in the plasma membrane and they dramatically reduce the effective intracellular concentration of xenobiotics or drugs. The pumps belong to either of two large protein families, the ATP-binding cassette (ABC) (CaCdr1p and CaCdr2p) or the major facilitator superfamily (MFS) (CaMdr1p) transporters. Much can be learned about how these proteins contribute to fungal drug resistance by expressing the proteins in a surrogate host.

We have created a heterologous membrane protein expression system using the eukaryotic model organism, *Saccharomyces cerevisiae*, and have successfully characterized Erg11p and multidrug efflux pumps from a number of different pathogenic fungi (6–8). This chapter describes the molecular biological steps necessary to achieve high levels of expression of functional CaCdr1p, the major efflux pump of clinically azole-resistant *C. albicans* isolates, or to achieve high levels of expression of C-terminally tagged CaCdr1p derivatives for purification or localization purposes.

2. Materials

Materials such as centrifuges (microfuges or standard centrifuges), water baths, gel electrophoresis equipment, a laminar flow unit in which to pour agar plates and to undertake other sterile work, a fume hood to protect the experimenter from toxic or carcinogenic fumes, a UV transilluminator with an attached camera and computer for the purpose of taking images of ethidium bromide (EtBr)-stained DNA agarose gels or Coomassie Brilliant Blue R250-stained protein polyacrylamide gels, constant temperature incubators, PCR machines, spectrophotometers, a set of pipettes and disposable plastic tips, reaction tubes (microfuge tubes, PCR tubes, and Falcon tubes), a water purification (e.g., Milli-Q) system to purify water to molecular biology grade (simply referred to in this chapter as H₂O), and other equipments are standard to any modern molecular biology laboratory and are not mentioned specifically in this chapter. There is a wide range of these products and materials available, and each laboratory will have its own preferences.

2.1. Isolation of Genomic DNA (gDNA) from *C. albicans* or Related *Candida* Species

1. YPD medium, routinely used to grow yeast cells, consists of 1% (w/v) Bacto-yeast extract (Difco Laboratories, Detroit, MI), 2% (w/v) Bacto-peptone (Difco), and 2% (w/v) glucose. YPD agar plates contain 2% (w/v) Bacto-agar (Danisco, New Zealand Ltd). YPD medium and YPD plates can be stored at 4°C for at least 6 months.
2. SCE-buffer for the digestion of the yeast cell wall: 1 M sorbitol, 60 mM EDTA, 100 mM Na-Citrate; pH 7.0. This buffer is autoclaved and stable when stored at room temperature (RT).
3. The zymolyase solution (10 mg/mL in SCE buffer; Zymolyase[®] 100T, Seigaku Corp., Tokyo, Japan) is best made fresh when needed (*see* **Note 1**).
4. The Qiagen DNeasy kit for animal tissue preparation (Qiagen Pty Ltd, Clifton Hill, Victoria, Australia) is used to isolate gDNA from *C. albicans* cells that, first, have had their cell wall removed through zymolyase treatment (*see* **Note 2**). It contains gDNA-binding columns, DNA binding and elution buffers, and an RNase A stock solution.

2.2. Cloning of *CaCDR1* into Plasmids *pABC3* or *pABC3-tag*

1. DNA oligomer primers (Sigma-Aldrich, Australia) of less than 40 nucleotides in length can be ordered as “ready desalted” quality (*see* **Note 3**).
2. All solutions (25 mM MgSO₄, 2 mM dNTPs, and 10× KOD⁺ buffer) required for the PCR amplification of

different *CaCDRI* open reading frame (ORF) fragments are provided by the KOD⁺ DNA polymerase manufacturer (Novagen, San Diego, CA).

3. The QIAquick[®] PCR purification kit (Qiagen Pty Ltd) is used to column purify PCR products.
4. EtBr (2 mg/mL) that is used to visualize DNA under UV light is highly carcinogenic (*see Note 4*) and light sensitive. Keep wrapped in an aluminium foil and store at RT.
5. 50× TAE (Tris, acetate, EDTA) that is used to make 1× TAE for DNA agarose gel electrophoresis consists of 2 M Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol), 1 M acetic acid, and 50 mM EDTA (disodium ethylenediamine-tetraacetate).
6. Ethanol (EtOH) that is used to wash or precipitate DNA is of molecular biology grade (Merck Ltd, Auckland, New Zealand).
7. Restriction enzymes and corresponding 10× buffer stocks and 10× bovine serum albumin (BSA; 1 µg/µL) stocks are from New England Biolabs (NEB; Beverly, MA) and stored at −20°C (*see Note 5*).
8. A description of the buffers recommended for individual restriction enzyme digests can be found in the appendix of any NEB catalogue.
9. The 10× DNA loading dye, that is used for the loading of DNA samples onto a DNA agarose gel, contains one or two dyes that are used to help visualize the progress of the gel electrophoresis (25 mg/L bromophenol blue and/or 25 mg/L xylene cyanol FF) dissolved in 50% glycerol to help the DNA sink into the gel loading slots.
10. 2.5 µL 1 kb plus DNA ladder (0.1 µg/µL in 1× DNA loading dye; Invitrogen Ltd, Auckland, New Zealand) is used routinely as a size marker and to quantify unknown amounts of DNA fragments separated by DNA agarose gel electrophoresis.
11. The QIAquick[®] gel extraction kit (Qiagen Pty Ltd) is used to isolate DNA fragments from an agarose gel after their separation by gel electrophoresis.
12. To prepare a 2× ligation buffer combine 200 µL of a 10× ligation buffer (300 mM Tris-HCl, pH 7.8, 100 mM MgCl₂; stable when stored at RT) with 200 µL 100 mM dithiothreitol (DTT; stable for at least 1 year when stored at −20°C), 200 µL of a 10× ATP stock (10 mM ATP, pH 7.0; adjust the pH to 7.0–7.5 with NaOH to keep the ATP stable in solution for at least 1 year when stored at −20°C), 350 µL of 40% PEG-6000 (w/v) (the

PEG solution is filter-sterilized with a 0.8- μ m Millipore membrane filter and can be stored for at least 1 year at -20°C , and 50 μL H_2O . The $2\times$ ligation buffer is stable and can be stored for at least 1 year at -20°C or -80°C .

13. Any T4 DNA ligase [e.g., T4 DNA ligase (1 U/ μL) from Roche Diagnostics NZ Ltd, Auckland, New Zealand] can be used for the ligation reaction (*see* **Note 6**).
14. SOB medium: 2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 10 mM NaCl, and 2.5 mM KCl. Autoclave and store at 4°C (stable for several months).
15. SOC medium is made fresh just before use: add 10 mL of a 2 M, filter-sterilized, magnesium stock solution (1 M $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ and 1 M $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$; kept at RT) to 1 L of SOB medium.
16. TB medium (10 mM HEPES, 15 mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 250 mM KCl, and 55 mM MnCl_2 ; pH 6.7) is made fresh just before use by mixing two separately autoclaved stock solutions (for an explanation, *see* **Note 7**).

Stock solution one: dissolve 2.38 g HEPES, 2.21 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 18.64 g KCl – adjust the pH to 6.7 with KOH – in a final volume of 945 mL, autoclave and store at 4°C .

Stock solution two: 1 M MnCl_2 , autoclave and store at RT.

17. DMSO is of molecular biology grade from Merck Ltd
18. Plasmids are maintained in *Escherichia coli* strain DH5 α .
19. *E. coli* cells are grown in Luria–Bertani (LB) medium [1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 1% (w/v) NaCl; pH 7.5], to which ampicillin (100 $\mu\text{g}/\text{mL}$; LBamp medium; *see* **Note 8**) is added as required. LB and LBamp agar plates contain 2% (w/v) Bacto-agar (Danisco, New Zealand Ltd).
20. Plasmids are isolated from individual *E. coli* transformants using a QIAprep[®] spin miniprep kit (Qiagen Pty Ltd).
21. Plasmids are sequenced with the DYEnamic ET terminator cycle sequencing kit v 3.1 (GE Healthcare UK Ltd, Buckinghamshire, UK) using the services of a DNA sequencing facility.

2.3. Transformation of *S. cerevisiae* AD Δ

1. Yeast cells were transformed using the alkali-cation yeast transformation kit from Bio 101 (Vista, CA).
2. YPAD medium is YPD medium supplemented with adenine (50 mg/L).
3. TE buffer (pH 7.5), lithium/cesium acetate, PEG, and TE/cation MIXX are part of the alkali-cation yeast transformation kit and stable at RT for many months.

4. Carrier DNA (20 $\mu\text{g}/\mu\text{L}$ salmon sperm DNA), histamine, and SOS medium are also supplied with the alkali-cation yeast transformation kit and must be stored at 4°C.
5. CSM-ura plates consist of 0.079% (w/v) complete supplement mixture without uracil (CSM-ura; Bio 101), 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 2% (w/v) glucose, and 2% (w/v) Bacto-agar (Danisco, New Zealand Ltd). Plates are stored at 4°C and stable for at least 6 months.

2.4. Confirmation of Positive *S. cerevisiae* Transformants by Colony PCR

1. Use TaKaRa Ex TaqTM DNA polymerase (5 U/ μL) and the buffers and solutions supplied for yeast colony PCR.
2. For the isolation of gDNA from single yeast colonies the Y-DERTM yeast DNA extraction reagent kit (Pierce, Rockford, IL) is used.
3. Isopropanol and a 75% EtOH solution (both are of molecular biology grade) are used for the EtOH precipitation of gDNA. All other solutions are provided by the manufacturer of the Y-DERTM yeast DNA extraction reagent kit and stored at 4°C. The solutions are stable for at least 1 year.

3. Methods

The following steps are required for the functional overexpression of the *C. albicans* multidrug efflux pump CaCdr1p in *S. cerevisiae* AD Δ (a graphical summary of the process is shown in **Fig. 15.1**): (1) isolation of gDNA from *C. albicans*; (2) PCR amplification of the *CaCDR1* ORF from gDNA; (3) digestion of the *CaCDR1* ORF with restriction endonucleases *PacI* and *NotI*; (4) gel purification of the *PacI*/*NotI*-digested ORF; (5) ligation of the ORF into plasmid pABC3 (or pABC3-tag); (6) transformation of *E. coli* with the ligation products; (7) isolation of plasmid DNA from individual *E. coli* transformants and identification of transformants containing a plasmid with the expected ORF; (8) confirmation of the ORF sequence by DNA sequencing; (9) isolation of the transformation cassette from the confirmed plasmid construct; (10) transformation of *S. cerevisiae* AD Δ with the transformation cassette; and (11) screening for, and selection of, positive yeast transformants by colony PCR.

3.1. Isolation of gDNA from *C. albicans* or Related *Candida* Species

1. Streak a frozen stock of wild-type *C. albicans* cells (e.g., a thick suspension of logarithmically grown *C. albicans* ATCC 10261 cells stored at -80°C in 1 mL aliquots of YPD medium supplemented with 25% glycerol) onto a

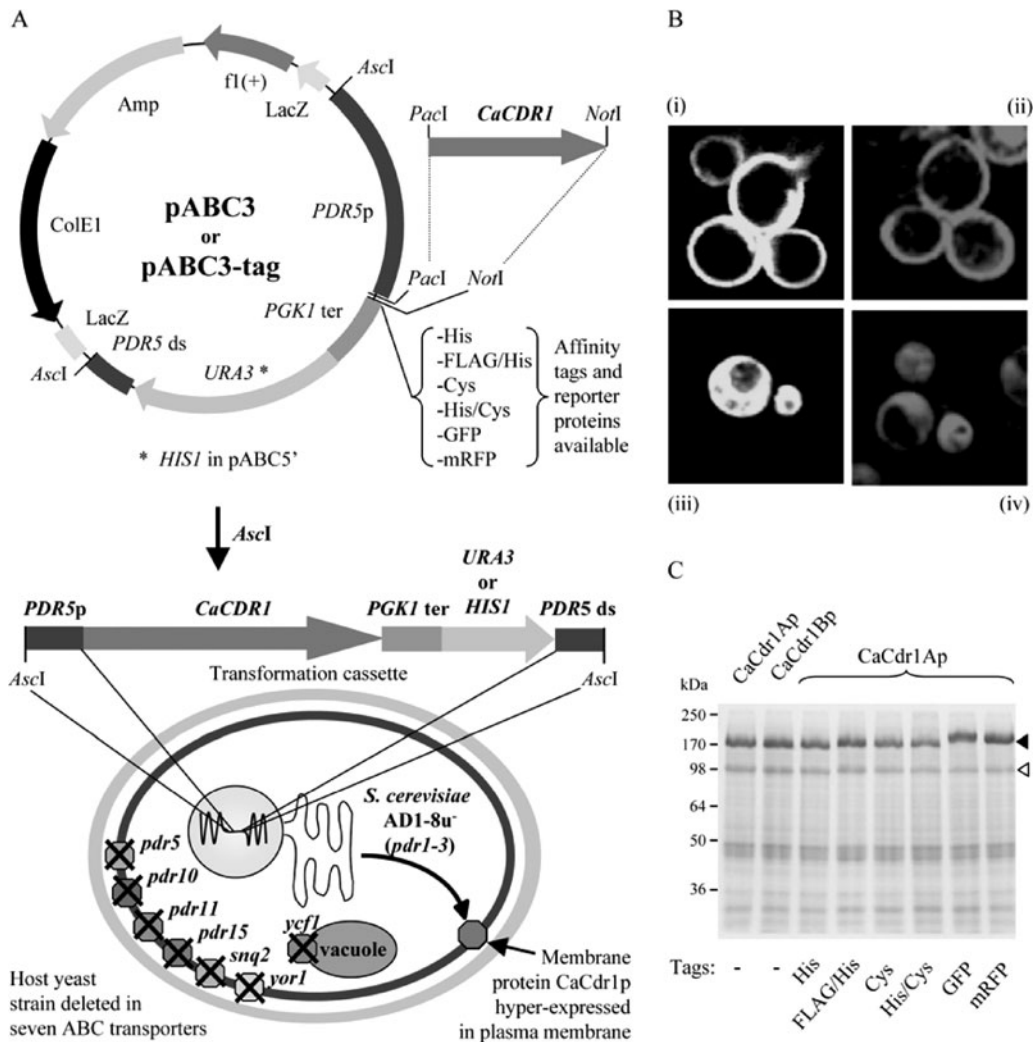


Fig. 15.1. **A** Schematic diagram of the yeast membrane protein hyper-expression system. The system comprises a set of vectors based on pBluescriptSK(+) containing a transformation cassette that consists of the *PDR5* promoter (dark grey), the *PGK1* terminator (medium grey), a selection marker (light grey) [*URA3* (pABC3 series of vectors) or *HIS1* (pABC5' series of vectors)], and a small part of the 3' end of the *PDR5* gene (dark grey). Unique 8 bp *PacI* and *NotI* cloning sites are located between the *PDR5* promoter and the *PGK1* terminator for the directional cloning of any ORF. Plasmids pABC3 and pABC5' provided templates for derivative plasmids that contain a choice of six different C-terminal tags located 3' to the *NotI* cloning site (pABC3-tag and pABC5'-tag). The transformation cassette containing an ORF cloned into the *PacI* and *NotI* sites can be excised as an *Ascl* fragment and used to transform the host strain *AD1-8u⁻*. **B** Confocal microscopy images of *AD1-8u⁻* cells over-expressing either the azole drug pump *CaCDR1p* (i) and (ii) or the cytosolic protein *CaUra3p* (iii) and (iv) tagged with either green (GFP) (i) and (iii) or red (mRFP) (ii) and (iv) fluorescent protein. **C** PM proteins (30 μ g/lane) of *AD1-8u⁻* cells hyper-expressing *CaCDR1Ap*, *CaCDR1Bp*, or *CaCDR1Ap* C-terminally tagged with each of the six tags were separated by SDS-PAGE and visualized with Coomassie Blue. Reproduced from *Eukaryotic Cell*, July 2007;6 (7):1150–65, DOI: 10.1128/EC.00091-07 with permission from the American Society for Microbiology (license number: 2102980481987).

- fresh YPD agar plate and incubate the plate at 30°C for 24–48 h for single colonies to appear.
2. Inoculate 10 mL YPD medium with a single yeast colony and grow the culture overnight (o/n) at 30°C for 16 h in a shaking incubator at 250 rpm to late logarithmic growth phase (*see* **Note 9**).
 3. Transfer 1–3 mL of yeast cells ($\sim 1 \times 10^8$ – 3×10^8 cells; *see* **Note 10**) into a 1.5 mL microfuge tube and harvest cells at maximum speed in a laboratory microcentrifuge for 1 min at RT.
 4. Resuspend the cell pellet with a pipetter in 600 μ L SCE-buffer that contains 1 μ L β -mercaptoethanol (~ 14 mM) (*see* **Note 11**).
 5. Add 10 μ L Zymolyase solution to the cell suspension and incubate at 37°C for 1–2 h with occasional gentle resuspension of cells by flicking and inverting the tube until the cell suspension becomes visibly viscous (do not vortex to avoid breakage of cells).
 6. Harvest cells by centrifugation at 5,000 rpm ($\sim 2,300g$) for 5 min (do not spin cells at higher g forces to avoid breakage of cells; *see* **Note 12**).
 7. Remove the supernatant with a 1 mL pipetter (*see* **Note 12**).
 8. Use the protocol of a commercially available gDNA extraction kit such as DNeasyTM tissue kit for animal tissue preparation to isolate gDNA from the pellet.
 9. Dissolve the final gDNA preparation in 100–200 μ L of the elution buffer (10 mM Tris–Cl, pH 7.0) supplied with the kit.
 10. Following this protocol the final gDNA concentrations range between 50 and 200 ng/ μ L.

3.2. Cloning of *CaCDR1* into Plasmid *pABC3* or *pABC3-tag*

3.2.1. DNA Oligomer Primer Design to Clone *CaCDR1* into *pABC3*

The *CaCDR1* ORF is amplified by PCR from *C. albicans* gDNA using a 5' primer containing a *PacI* restriction enzyme site and a 3' reverse primer containing a *NotI* restriction enzyme site immediately downstream of the *CaCDR1* ORF stop codon (**Fig. 15.1A**). For cloning purposes, and in order to obtain the highest possible levels of *CaCDR1* expression, the 5' primer contains the *PacI* recognition site 11 bp upstream of the ATG start codon followed by AAA and then the *CaCDR1* ATG start codon. This maintains the size of the 5' untranslated region of the *PDR5* mRNA leader sequence and, most importantly, preserves

the Kozak consensus sequence for highly expressed genes in *S. cerevisiae*, particularly the A at position-3 (shown in bold font and underlined). The 3' reverse primer is designed so that the ORF sequence ends with the stop codon **TAA** followed by two nucleotides, AT, to ensure efficient termination of highly expressed genes, followed by the *NotI* restriction site. Three extra nucleotides (any suitable random sequence NNN) are added at the 5' ends of both primers to ensure efficient cutting of the amplified *CaCDR1* ORF fragments with *PacI* and *NotI*. The design of the primers to amplify the *CaCDR1* ORF is therefore as follows:

1. 5' primer: 5'-NNN TTAATTAA AAA ATG plus the first 20 bp of the chosen ORF.
2. 3' primer: 5'-NNN GCGGCCGC AT TTA plus the reverse complement sequence of the last 20 bp of the *CaCDR1* ORF (see **Note 13**).

3.2.2. DNA Oligomer Primer Design to Clone *CaCDR1* into pABC3-tag

Directional cloning of *CaCDR1* or any other ORFs as *PacI*/*NotI* fragments into any of the six pABC3 derivative plasmids with a choice of different C-terminal tags (pABC3-His, pABC3-Cys, pABC3-His/Cys, pABC3-Flag/His, pABC3-Gfp, or pABC3-mRfp; **Fig. 15.1**) requires a modification of the 3' primer described above in **Section (3.2.1)**. The modification includes the removal of the AT TTA reverse stop codon sequence and its replacement with one extra C nucleotide to ensure an in-frame C-terminal fusion of the ORF with the chosen tag. Thus, the 3' reverse primer sequence to PCR amplify the *CaCDR1* ORF for cloning into any of the six derivative plasmids has the following sequence: 5'-NNN GCGGCCGC C plus the reverse complement sequence of the last 20 bp of the *CaCDR1* ORF (see **Note 14**).

3.2.3. PCR Amplification of *CaCDR1* ORFs from *C. albicans* gDNA

1. To amplify *CaCDR1* use a high-fidelity proof reading DNA polymerase such as KOD⁺ DNA polymerase (see **Note 15**).
2. Set up 50 μ L PCR reactions in 0.5 mL PCR tubes by mixing the following components in the given order:

25.6 μ L	H ₂ O
5 μ L	10 $\times$ KOD ⁺ buffer
2.4 μ L	MgSO ₄ (25 mM)
5 μ L	dNTPs (2 mM each)
5 μ L	5' primer (3 μ M)
5 μ L	3' primer (3 μ M)
1 μ L	gDNA ($\approx$ 100 ng/ μ L)
1 μ L	KOD ⁺ DNA polymerase (1 U/ μ L)
3. Mix thoroughly by pipetting up and down three times with a pipetter that has been set to 50 μ L. Checking the volume

that way will also confirm that none of the components are missing.

4. Make sure that the tubes are closed properly and put them into the heating block of a PCR machine (thermal cycler).
5. Close the lid of the PCR machine and start the following PCR protocol (*see* **Note 16**):

	94°C	5 min
34×	→ 94°C	20 s
	55°C	10 s
	68°C	5 min (~ 1 min/kb ; <i>CaCDR1</i> is ~ 4.5 kb)
	68°C	10 min
	4°C	hold

6. Run a 1 μ L portion of the above PCR (plus 8 μ L H₂O and 1 μ L 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel containing EtBr (*see* **Note 17**) to check the quality and amount of the amplified DNA fragment (*see* **Note 18**).

3.2.4. Digestion of *CaCDR1* PCR Fragment with *PacI* and *NotI*

3.2.4.1. Purification of Amplified PCR Products with Spin Columns

1. To be able to digest the amplified *CaCDR1* ORF with *PacI* and *NotI*, a buffer change is needed. This can be achieved by spin column-based purification of the PCR fragment using a QIAquick[®] PCR purification kit (*see* **Note 19**).
2. To column purify the remaining 49 μ L of PCR product (after 1 μ L has been run on an agarose gel in Step 6 of **Section 3.2.3**) follow the instructions of the kit manufacturer.
3. In short, 49 μ L of the PCR is mixed with five volumes ($5 \times 49 \mu\text{L} = 245 \mu\text{L}$) of PB buffer (binding buffer) and loaded onto a small spin column. The *CaCDR1* PCR product is bound to the column by spinning the solution through the column at maximum speed in a microfuge.
4. Collect the flow-through and discard it into a waste container.
5. Wash the bound DNA with 500 μ L wash buffer (*see* **Note 20**).
6. Elute the bound DNA into a 1.5 mL microfuge tube. For maximum recovery of the bound DNA, elute twice with

30 μL elution buffer, instead of only once with 50 μL , as recommended by the kit manufacturer.

7. Separate 1.2 μL of the purified PCR fragment (plus 8 μL H_2O and 1 μL 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel to estimate the DNA recovery of the purified product (*see* **Note 21**).

3.2.4.2. Digestion of *CaCDR1* with *PacI* and *NotI*

1. Digest the purified *CaCDR1* fragment with *PacI* (*see* **Note 22**). To do so, add the following ingredients to the remaining 58.8 μL purified *CaCDR1* PCR fragment from step 7 above (**Section 3.2.4.1**) in the given order:

4.7 μL H_2O
 8 μL 10 $\times$ NEB buffer 1
 8 μL 10 $\times$ BSA (1 $\mu\text{g}/\mu\text{L}$)
 0.5 μL *PacI* (10 U/ μL)

2. Mix the reaction by pipetting up and down and incubate at 37°C for 2 h.
3. Add an extra 0.5 μL of *PacI* enzyme (*see* **Note 23**).
4. Incubate at 37°C for a further 1 h.
5. Purify the *PacI*-digested *CaCDR1* PCR fragment using the QIAquick® PCR purification kit (as described in **Section 3.2.4.1**).
6. Separate 1.2 μL of the *PacI*-digested and spin column-purified PCR fragment (plus 8 μL H_2O and 1 μL 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel (again, for an estimation of DNA recovery compare the intensity of the EtBr-stained DNA band with the band intensity of 1.2 μL of the column-purified PCR product in Step 7 of **Section 3.2.4.1**).

7. Digest the purified *CaCDR1* fragment with *NotI*. To do so, add the following ingredients to the remaining 58.8 μL purified, *PacI*-digested, *CaCDR1* PCR fragment in the given order:

3.7 μL H_2O
 7 μL 10 $\times$ NEB buffer 3
 0.5 μL *NotI* (10 U/ μL)

8. Mix by pipetting up and down and incubate at 37°C for 2 h.
9. Add an extra 0.5 μL of *NotI* enzyme (*see* **Note 23**).
10. Incubate at 37°C for a further 1 h.

3.2.4.3. Gel Purification of DNA Fragments

In order to successfully clone *CaCDR1* into vector pABC3 or pABC3-His it is absolutely essential that the *PacI*/*NotI*-digested *CaCDR1* ORFs be gel purified. Failure to do so will result in cloning only empty vector (*see* **Note 24**).

1. After the *NotI* digest has been completed add $\sim 8 \mu\text{L}$ of $10\times$ DNA loading dye, mix the solution with a pipetter and load about 90% ($\sim 70 \mu\text{L}$) into a large double lane (*see below*) of a 0.7% DNA agarose gel, or a triple lane if the double lane will not hold $70 \mu\text{L}$, and load the rest ($\sim 8 \mu\text{L}$) of the solution into an adjacent single lane. To create a double or triple lane use masking tape to tape two teeth (or three if necessary) of a comb together before pouring the agarose gel.
2. Run the gel at 100 V for 1–1.5 h to separate the *CaCDRI* amplicon from the small *PacI* and *NotI* adapters.
3. Isolate the *CaCDRI* fragment by cutting the band out of the gel using a UV transilluminator that visualizes EtBr-stained DNA bands. *Danger*: UV light is a potent mutagen – do not expose DNA (or your skin) at any time to UV light (*see Note 25*). To avoid any exposure of DNA to UV light, cut the agarose gel (containing EtBr for DNA staining; *see Note 17*) into two strips and only expose the single lane, that contains just 10% of the sample, to UV light, and mark where the top and bottom of the *CaCDRI* band are with a scalpel. Then reassemble the agarose gel strips and use the marked incisions as a guide to cut the remaining 90% of *CaCDRI* out of the agarose gel. For the purpose of gel purification, make sure to cut as small a band as possible (*see Note 26*).
4. Collect the excised bands into pre-weighed 1.5 mL microfuge tubes and calculate the weights of the agarose gel slices.
5. Then extract the DNA from the excised gel slices using a QIAquick[®] gel extraction kit (*see Note 27*) following the manufacturer's instructions.
6. In short, add three volumes of QG extraction buffer to one volume of gel slice and dissolve the agarose gel by putting the closed tube into a 50°C water bath for 10–20 min. Make sure that the gel slice is fully dissolved before proceeding with the next step.
7. Add one volume of isopropanol and mix by inverting the closed tube. This step helps increase the yield of DNA and should always be included.
8. Bind the DNA onto a gel extraction column (the columns are the same as those used for the PCR purification) by loading and spinning the solution through the column at maximum speed in a microfuge for about 30 s. Repeat the procedure until all the DNA solution is bound to the column.

9. The washing and elution steps are the same as described above for the PCR purification protocol (Steps 5–7 of **Section 3.2.4.1**).
10. Run 1.2 μL of the 60 μL eluted DNA (plus 8 μL H_2O and 1 μL 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel to estimate the DNA recovery (*see Note 28*) of the final *PacI*/*NotI*-digested, and gel purified, *CaCDRI* PCR product (compare the intensity of the EtBr-stained DNA band with the band intensity of the column purified, *PacI*-digested, PCR product of Step 6 of **Section 3.2.4.2**).

**3.2.5. Preparation
of *PacI*/*NotI*-Digested
Plasmids pABC3
and pABC3-His**

To ligate and clone *CaCDRI* into pABC3 or pABC3-His, each plasmid needs to be completely digested with both *PacI* and *NotI* restriction endonucleases to prevent the plasmids from self-ligating, rather than with the *CaCDRI* insert, in the subsequent DNA ligation reaction (*see Note 29*).

1. Cut 2 μg of each plasmid in a total volume of 50 μL with *PacI*. Take 10 μL of each plasmid stock (200 ng/ μL ; *see Note 30*), add 5 μL of 10 $\times$ NEB buffer 1, 5 μL 10 $\times$ BSA stock, and 29 μL H_2O , and start the restriction digest by adding 0.5 μL of *PacI* enzyme (10 U/ μL) and incubating the reaction at 37°C for 2 h.
2. Add an additional 0.5 μL of *PacI* and incubate for another 1 h at 37°C to make sure that the plasmids are cut to completion (*see Note 23*).
3. Run 1 μL (plus 8 μL H_2O and 1 μL 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel as a control to check that *PacI* has digested the plasmid to completion. Uncut, freshly prepared, plasmid DNA runs faster than digested, and therefore, linear plasmid DNA and can easily be distinguished on an agarose gel (*see Note 31*).
4. Column purify the *PacI*-digested plasmids and cut the plasmids with *NotI* in a total volume of 80 μL , using the procedure employed to prepare *PacI*/*NotI*-digested *CaCDRI* fragments as described above in **Section 3.2.4.2** (*see Note 32*).
5. Gel purify the *PacI*/*NotI* double digested plasmids pABC3 and pABC3-His together with the above prepared, *PacI*/*NotI*-digested *CaCDRI* fragments (as described in **Section 3.2.4.3**).
6. Run 1 μL of each purified DNA fragment (plus 8 μL H_2O and 1 μL 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel as a control and to estimate the DNA concentrations (*see Note 33*) and store them at -20°C .

3.2.6. DNA Ligation

For an optimum ligation choose a $\sim 10\times$ molar excess of insert (*CaCDRI*) over plasmid molecules pABC3 or pABC3-His (to estimate amounts of DNA, *see* **Note 33**).

1. Warm the $2\times$ ligation buffer and all other ingredients to RT before use (*see* **Note 34**).
2. Set up the ligation reactions (*see* **Note 35**) at RT by mixing the following ingredients:

9-(x+ y) μL	H ₂ O
x μL	insert (50–100 ng <i>CaCDRI</i> <i>PacI</i> / <i>NotI</i> -digested)
y μL	plasmid (5 ng pABC3 or pABC3-His; each <i>PacI</i> / <i>NotI</i> -digested)
10 μL	$2\times$ ligation buffer
1 μL	T4 DNA ligase (1 U/ μL)
3. Incubate at RT for 1 h (*see* **Note 36**).
4. Store the ligation reactions at 4°C until needed (*see* **Note 37**).

3.2.7. Transformation of *E. coli*

These ligation reactions can now be used to transform *E. coli* DH5 α for the propagation of individual ligated plasmids. *E. coli* transformants will recover in SOC medium supplemented with glucose for 1 h, and aliquots are then plated onto selective medium. Only *E. coli* cells that have been successfully transformed can grow on the selective medium (LBamp).

3.2.7.1. Creating Highly Competent *E. coli* Cells According to Inoue et al.

1. Inoculate 250 mL SOB medium with three colonies of *E. coli* DH5 α cells grown o/n on an LB plate.
2. Grow the cells with shaking at 250 rpm at $18\text{--}22^\circ\text{C}$ to an OD₆₀₀ of 0.6 [takes about 1.5–2 days; cells grow very slow at 18°C (estimated generation time is ~ 6 h)].
3. Place flask on ice for 10 min.
4. Transfer cells to a 250 mL ice-cold, sterile, centrifuge tube and harvest the cells by centrifugation with a large Sorvall F14S or similar rotor at 3,000 rpm ($\sim 1,380g$) for 10 min at 4°C .
5. Resuspend the cell pellet in 80 mL of ice-cold, freshly prepared, TB buffer (*see* **Note 38**).
6. Keep the cells on ice for 10 min.
7. Harvest the cells again at $\sim 1,380g$ in the same rotor for 10 min at 4°C .
8. Resuspend the cells gently in 20 mL ice-cold TB buffer.
9. Add 1.5 mL DMSO slowly (dropwise, takes about 5 min) with gentle swirling of the cell suspension to a final concentration of 7% DMSO.

3.2.7.2. Transformation of *E. coli* DH5 α with Ligation Mix

10. Incubate the cells on ice for at least a further 10 min.
11. Aliquot the competent cells into pre-cooled 1.5 mL microfuge tubes.
12. Place the highly competent cells (*see* **Note 39**) in a -80°C freezer for long-term storage (9).

1. Take the freshly prepared competent cells or thaw an aliquot of competent cells that were kept at -80°C , on ice.
2. Add 4 μL of ligation mix to an ice-cold 15 mL Falcon tube (*see* **Note 40**).
3. Add 200 μL of ice-cold competent cells to the ligation mix in the Falcon tube.
4. Gently mix the cells by flicking the Falcon tube and incubate the cells on ice for 30 min with periodic gentle mixing.
5. Heat-shock the cells for 45 s without agitation in a 42°C water bath.
6. Transfer the tube of *E. coli* DH5 α cells onto ice for 2 min.
7. Add 0.8 mL pre-warmed (42°C) SOC medium (*see* **Note 41**) to the transformed cells.
8. Let the cells recover by incubating them at 37°C for 1 h in a shaking incubator (250 rpm).
9. Pipette a 1–100 μL portion (*see* **Note 42**) of the transformed and recovered cells onto LBamp plates, spread them evenly with a glass rod until the plate surface is dry, and incubate the cells at 37°C o/n for *E. coli* transformants to appear.

Transformation with the control ligation mix containing only *PacI*/*NotI* double-digested plasmid (background negative control experiment) should only give a few transformants (1 to <100) while the transformation with the ligation mix (pABC3 plus *CaCDRI*) should give 10- to 1,000-fold more transformants (*see* **Note 43**).

3.2.8. Isolation of Plasmid DNA from *E. coli* Transformants

Plasmid preparations are made from individual *E. coli* transformants to identify those that contain the desired pABC3-*CaCDRI* plasmid. If significantly more transformants were obtained in the transformation with the ligation mix that contained vector (pABC3) plus insert (*CaCDRI*) compared to the control transformation with the ligation mix that contained vector (pABC3) only, then the majority of transformants should contain the desired plasmid. If, however, there was no increase in the number of transformants for the experimental plate compared to the control plate, determine which of the previous DNA manipulation steps failed (for possible reasons of failure, *see* **Note 43**). If the controls show that the experiment worked well, isolate plasmid DNA from 5 to 10 *E. coli* transformants.

1. Pick a single *E. coli* colony with a sterile toothpick and inoculate 3 mL portions of LBamp medium in sterile plastic tubes by simply adding the toothpick to the medium.
2. Grow cells at 37°C in a shaking incubator at 250 rpm o/n.
3. Harvest the cells by spinning the plastic tubes in a lab centrifuge at 7,000 rpm (~6,030*g*) for 5 min at RT.
4. Resuspend the cells in the recommended amount of buffer P1 (QIAprep[®] spin miniprep kit), transfer the cells to a 1.5 mL microfuge tube, and follow the manufacturer's instructions to isolate the plasmid DNA.
5. Elute the plasmid DNA from the DNA binding column with 50 µL elution buffer and store at -20°C (*see Note 44*).

3.2.9. Identification of Plasmids Containing *CaCDR1* by Mapping Restriction Sites

Perform a diagnostic digest of 1 µL of each plasmid DNA mini-prep either with *NotI*, *PacI*, or *AscI* to identify plasmids that contain *CaCDR1*. A diagnostic digest is performed in a total volume of 15 µL with about 1–2 U of restriction enzyme (*see Note 45*).

1. Make a 6× master mix that contains all the ingredients, except 1 µL plasmid DNA, that are needed for the restriction digest of five plasmid mini-preps (*see Note 46*). For example, for a *PacI* digest of five plasmid DNA mini-preps:

1 × mix	6 × master mix
10.9 µL	65.4 µL H ₂ O
1.5 µL	9 µL 10× NEB buffer 1
1.5 µL	9 µL 10× BSA (1 µg/µL)
0.1 µL	0.6 µL <i>PacI</i> (10 U/µL)
2. Put 1 µL of each plasmid DNA mini-prep into a 1.5 mL microfuge tube.
3. Add 14 µL of the master mix to each of the five tubes and mix by pipetting up and down.
4. Incubate at 37°C for 1 h.
5. Add 2 µL 10× DNA loading dye and separate digested DNA on a 0.7% DNA agarose gel. pABC3-*CaCDR1* is 10.2 kb in size and is cut once with *PacI*, once with *NotI*, and twice with *AscI* (resulting in two fragments: 2.7 and 7.5 kb).
6. Identify *E. coli* clones containing potentially correct plasmids (showing the expected restriction pattern) and confirm one of these plasmids as correct by sequencing the whole *CaCDR1* ORF.

3.2.10. Sequencing *pABC3-CaCDR1*

1. To sequence the whole *CaCDR1* ORF design DNA oligomer primers [20 nucleotides; (*see Note 47*)] specific to *CaCDR1* positioned every 600 bp.

2. Send sequencing primers together with the column-purified candidate plasmid pABC3-CaCDR1 to a professional sequencing facility.
3. Keep at least two further candidate pABC3-CaCDR1 plasmid mini-preps as back-ups in case the first plasmid DNA sequence is not 100% correct (*see* **Notes 48** and **49**).

3.2.11. Isolation and Purification of the CaCDR1 Transformation Cassette

To create a *S. cerevisiae* AD Δ strain that overexpresses CaCdr1p the transformation cassette must be isolated from pABC3-CaCDR1 by digesting the plasmid with the *AseI* restriction enzyme, gel purified, and then used to transform AD Δ .

1. Digest 2 μ g of pABC3-CaCDR1 with *AseI* in an appropriate volume (*see* **Note 50**) to release the transformation cassette as a linear piece of DNA (*see* **Note 51**).
2. Run 1 μ L (plus 8 μ L H₂O and 1 μ L 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel to test whether the DNA has been completely digested.
3. Separate the DNA in the remainder of the digest on a 0.7% DNA agarose gel and extract and gel purify the larger ($\sim$ 7.5 kb) fragment containing the linearized transformation cassette from the gel as described above (**Section 3.2.4.3**).
4. Check 1 μ L of the gel-purified fragment (plus 8 μ L H₂O and 1 μ L 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel and quantify the concentration of the fragment by comparison to the DNA size marker (*see* **Note 33**).

3.3. Transformation of *S. cerevisiae* AD Δ

The integration of the linearized transformation cassette occurs at the chromosomal *S. cerevisiae* *PDR5* locus via homologous recombination at both ends of the cassette (the 5' end contains the *PDR5* promoter and the 3' end contains part of the *PDR5* terminator; **Fig. 15.1A**). Like *E. coli* cells, *S. cerevisiae* cells have to be treated to be competent (i.e., able to take up DNA). In a first step, *S. cerevisiae* cells are made competent and in the second step they are heat-shocked for 10 min to induce them to take up the DNA and integrate it into their chromosome via a homologous recombination event that is very specific and efficient in *S. cerevisiae*. All steps of the protocol are performed at RT unless otherwise stated.

3.3.1. Generating Competent *S. cerevisiae* AD Δ Cells According to Schiestl and Gietz

1. Streak AD Δ cells from a frozen YPD/glycerol stock onto a YPD agar plate and incubate at 30°C for 2 days until single colonies appear.
2. Inoculate 10 mL of YPD medium with a single colony of AD Δ cells and incubate at 30°C in a shaking incubator (250 rpm) o/n.

3. Take a portion of the o/n culture and inoculate 250 mL YPAD medium to an OD₆₀₀ of 0.1.
4. Grow the cells at 30°C with shaking at 250 rpm for 4–6 h to an OD₆₀₀ of ~0.6.
5. Transfer cells to a 250 mL, sterile, centrifuge tube and harvest the cells by centrifugation with a large Sorvall F14S or similar rotor at 5,000 rpm (~3,840*g*) for 3 min.
6. Wash the cells in 10 mL TE buffer (pH 7.5) and transfer to a 50 mL, sterile, centrifuge tube.
7. Harvest the cells by centrifugation with a small Sorvall F21 rotor at 5,000 rpm (~2,970*g*) for 1 min.
8. Resuspend the cells in 5 mL lithium/cesium acetate solution (alkali-cation yeast transformation kit from BIO 101).
9. Incubate the cells at 30°C for 25 min (and no longer; *see Note 52*) with gentle shaking (100 rpm).
10. Centrifuge the cells at ~2,970*g* for 1 min.
11. Resuspend the cells in 1 mL TE buffer (pH 7.5) by gently pipetting up and down.
12. The cells are now transformation competent (*see Note 53*), keep them on ice until they are needed for transformation with DNA.
13. Alternatively, the competent cells can be frozen for long-term storage by adding 1/3 volume of a sterile 50% glycerol stock and stored at –80°C (*see Note 54*) (10).

*3.3.2. Transformation of
S. cerevisiae ADΔ Cells
with the pABC3-CaCDR1
Transformation Cassette*

1. Pipette the required amount (5 μL per transformation) of carrier DNA (20 μg/μL salmon sperm DNA; stored at 4°C) into a 1.5 mL microfuge tube, close the lid (*see Note 55*), and denature the carrier DNA in a boiling water bath for 5 min (*see Note 56*).
2. Put the tube immediately on ice. The carrier DNA is now denatured and ready to be used for the transformation.
3. Combine the following on ice in a 1.5 mL microfuge tube and mix by pipetting up and down:

5 μL	Carrier DNA
5 μL	Histamine solution (<i>see Note 57</i>)
<i>x</i> μg	Plasmid DNA in a maximum volume of 10 μL
4. Add 100 μL competent ADΔ cells.
5. Gently mix by flicking the tube a few times and incubate at RT for 15 min with periodic gentle mixing.
6. Add 1 mL of PEG/TE/cation MIXX (alkali-cation yeast transformation kit from BIO 101; *see Note 58*).

7. Mix the cells in the solution by thoroughly flicking and inverting the tube (*see* **Note 58**).
8. Incubate the cells at 30°C for 10 min, then gently resuspend cells by flicking the tube.
9. Heat-shock the cells at 42°C in a water bath for 60 min.
10. Keep the tubes on the bench at RT for 2 min.
11. Centrifuge the cells in a microcentrifuge at maximum speed for 10 s and remove the supernatant with a 1 mL pipetter (*see* **Note 59**).
12. Resuspend the cells in 100 μ L SOS medium (mixing and pipetting up and down with a pipetter) and immediately plate the cells on selective medium (CSM-ura plates; *see* **Note 60**).
13. Incubate the plates at 30°C for 2–4 days for yeast colonies to appear.

3.4. Confirmation of Positive *S. cerevisiae* Transformants by Colony PCR

Unlike for bacteria, performing colony PCR on intact yeast cells (*S. cerevisiae* or *Candida* species) is difficult (*see* **Note 61**). We provide two relatively easy protocols that work reproducibly for *S. cerevisiae* and *Candida*. The first protocol is faster, uses intact cells, but is only suitable for the amplification of short PCR products (maximum size of amplified product is 2–3 kb), while the second protocol is more involved but generally applicable with no size limitation for the amplified PCR product.

3.4.1. Colony PCR with Takara Taq DNA Polymerase Using Intact Yeast Cells

This protocol is very simple but recommended for short, control, PCRs only. From experience, TaKaRa Ex TaqTM DNA polymerase allows the reliable and reproducible amplification of short PCR products from intact yeast cells while other commercially available Taq DNA polymerases give inconsistent results.

1. Set up on ice 10 μ L PCRs with TaKaRa Ex TaqTM DNA polymerase in 250 μ L PCR tubes. If many colonies need to be tested (e.g., 10) make a master mix (e.g., 11 times):

1 $\times$ mix	11 $\times$ mix	
6.5 μ L	71.5 μ L	H ₂ O
1 μ L	11 μ L	PDR5up primer (3.2 μ M; <i>see</i> Note 62)
1 μ L	11 μ L	CaCDRI-rev primer (3.2 μ M; <i>see</i> Note 62)
1 μ L	11 μ L	10 $\times$ Takara PCR buffer
0.4 μ L	4.4 μ L	dNTPs (10 mM each)
0.1 μ L	1.1 μ L	TaKaRa Ex Taq TM DNA polymerase (5 U/ μ L)

2. Dispense 10 μL aliquots of the master mix into 10 PCR tubes.
3. Label the $\text{AD}\Delta$ transformants to be tested, on the base of the agar plate, with a pen.
4. Touch each labeled $\text{AD}\Delta$ transformant with a pipette tip and mix the cells into the master mix in the PCR tube (*see Note 63*).
5. Run the following PCR protocol:

	94°C	5 min
34×	→ 94°C	20 s
	55°C	10 s
	72°C	1.0 min (~ 0.5 min/kb ; expected size ~ 2 kb)
	72°C	5 min
	4°C	hold

6. Separate 1–2 μL (plus 8 μL H_2O and 1 μL 10 $\times$ DNA loading dye) on a 0.7% DNA agarose gel to identify which $\text{AD}\Delta$ transformants contain the *CaCDRI* transformation cassette properly integrated at the 5' end of the genomic *PDR5* locus (expect a PCR product of ~2 kb for positive transformants; *see Note 62*).
7. Repeat the colony PCR for the same transformants with the DNA oligomer primer pair *PDR5*-down/*URA3*-for to confirm proper integration of the transformation cassette at the 3' end as well (expect a PCR product of ~1.0 kb; *see Note 62*).

3.4.2. Mini-gDNA Extraction from Single *S. cerevisiae* Colonies for Colony PCR

The isolation of gDNA from *S. cerevisiae* transformants is required for an alternative PCR protocol that is very reliable. The gDNA isolated from a single colony can be stored for a long time at -20°C and provides sufficient DNA template for at least 20 different PCR reactions. This protocol works equally well for older agar plates that contain yeast colonies that have been stored for long periods of time (weeks or even months at 4°C).

1. Pipette 10 μL Y-PER reagent (Y-DER gDNA extraction kit) into a 1.5 mL microfuge tube (*see Note 64*).
2. Stab a single $\text{AD}\Delta$ transformant from the CSM-ura selection plate with a 200 μL pipette tip and resuspend the cells in 10 μL Y-PER by pipetting up and down (*see Note 65*).
3. Incubate the cells at 65°C for 10 min.

4. Harvest the cells by centrifugation at maximum speed in a microcentrifuge.
5. Aspirate and discard the supernatant in an appropriate waste container.
6. Resuspend the cell pellet in 8 μ L DNA Releasing Reagent A (*see* **Note 66**).
7. Add 8 μ L DNA Releasing Reagent B and mix by pipetting up and down.
8. Incubate the cells at 65°C for 10 min.
9. Add 4 μ L of protein removal reagent and mix gently by pipetting up and down.
10. Centrifuge cell mixture at maximum speed in a microcentrifuge for 5 min.
11. Transfer the supernatant to a new 1.5 mL microfuge tube.
12. Add 12 μ L isopropanol and mix by pipetting up and down to precipitate the gDNA.
13. Spin the gDNA at maximum speed in a microfuge for 10 min.
14. Remove the supernatant and wash the gDNA pellet with 200 μ L 75% EtOH.
15. Spin the gDNA at maximum speed in a microfuge for 5 min.
16. Remove the supernatant carefully. Make sure to remove all EtOH.
17. Air-dry for a few minutes and resuspend the gDNA pellet (the pellet is not visible) in 20 μ L H₂O and store at -20°C.
18. Use DNA as template for a regular 10 μ L PCR to confirm individual AD Δ transformants. Although the concentration of gDNA is low and not visible on an agarose gel, 1 μ L provides enough, very clean, gDNA template to get good and reliable PCR products using any DNA polymerase in a regular PCR.
19. Use gDNA as template (1 μ L per reaction) and perform the two PCRs with the 5' and 3' DNA oligomer primer pairs as described in **Section 3.5.1** to confirm that AD Δ transformants contain the *CaCDR1* transformation cassette properly integrated at the chromosomal *PDR5* locus.

S. cerevisiae AD Δ /CaCDR1 cells can now be used for further biological tests such as measuring their increased levels of resistance to different drugs, for purification of CaCdr1-His with Ni⁺ affinity chromatography or for many other functional assays.

4. Notes

1. Zymolyase[®] 100T does not dissolve properly but rather remains as a suspension.
2. Before the Qiagen DNeasy kit can be used for the isolation of gDNA from *Candida* cells, their cell wall has to be removed with zymolyase treatment.
3. No chemically synthesized DNA oligomer primer is ever perfect. As a rough guide one can estimate that every cycle of DNA oligomer primer synthesis is only 99.5% accurate. Most of the mistakes introduced during the synthesis of primers are deletions (nonincorporation of nucleotides). Thus, for a primer of 25 nucleotides at least 10% ($25 \times \sim 0.5\%$) of the primer molecules can be expected to contain one or more mutations (mostly deletions). If the primers are larger than 40 nucleotides and need to be of high accuracy (e.g., when used for cloning purposes or the mutagenesis of particular amino acid sequences), order HPLC or, best of all, PAGE-purified primers!
4. EtBr is highly carcinogenic. Use gloves at all times when handling EtBr and thoroughly rinse with water all electrophoresis equipment that comes into contact with EtBr. Dispose of EtBr waste according to local regulations.
5. Most restriction enzymes are stable for at least 1 year and up to 5 years when stored at -20°C . Use a portable -20°C freezer box to keep restriction enzymes, and indeed all enzymes used for molecular biological experiments (e.g., DNA ligases, DNA polymerases, etc.), stable on the bench for short periods.
6. There is no need to use a high-concentration T4 DNA ligase.
7. MnCl_2 needs to be kept separately because it oxidizes relatively easy (it turns pink or even a darker brown color). Do not use MnCl_2 stocks that are more than slightly pink.
8. Ampicillin (Amp) is unstable. Add Amp (from a stock of Amp dissolved in H_2O , $100\text{ }\mu\text{g}/\mu\text{L}$, kept at -20°C) after autoclaving the medium and just before pouring the plates (the temperature of the medium should be no more than 45°C). Amp-containing media last for only a few weeks when stored in the dark at 4°C .
9. The estimated generation time for *Candida* species grown in YPD at 30°C is about 1 h, and cells generally reach late logarithmic growth phase after 16 h at which stage the

OD₆₀₀ of the culture is usually about 10 (a stationary phase culture of *Candida* cells reaches OD₆₀₀ values of 15–20).

10. Assume that 1 mL of a yeast culture (the term yeast, in this case, includes different *Candida* species, *S. cerevisiae*, and *C. neoformans*) with an OD₆₀₀ value of 1 contains $\approx 10^7$ cells/mL.
11. β -Mercaptoethanol is highly toxic and its removal from the bottle must be performed with care in a fume hood.
12. The cells at this stage are spheroplasts (cells with no cell wall) and therefore are very fragile. After the cells have been harvested gently at low speed to avoid the breakage of cells, the cell pellet is not very tight, and it is therefore difficult to take off the supernatant. Use a pipetter to remove most of the supernatant – it does not matter if some liquid remains on the pellet.
13. In rare cases, where the ORF contains an internal *PacI* or *NotI* site, the ORF PCR fragment should firstly be partially digested with the enzyme that cuts within the ORF (e.g., *PacI* for *PDR5*; a *CaCDR1* homolog in *S. cerevisiae*), then the full-length partially digested ORF fragment is gel purified and digested with the second enzyme (e.g., *NotI*), before cloning it (e.g., *PDR5*) into pABC3.
14. The 8 bp of the *NotI* restriction site together with the extra C nucleotide that connects the *CaCDR1* ORF with the C-terminal tag of the chosen vector (e.g., vector pABC3-His) creates an extra three amino acid linker between the CaCdr1p protein and the tag (e.g., His tag). After cloning *CaCDR1* into any of the pABC3-tag plasmids, it creates 5'-*CaCDR1*-ORF-**G**GC-GGC-CGC-tag-3' or translated: CaCdr1p-gly-gly-arg-tag (the **G** in bold type is derived from the extra **C** that was inserted into the 3' primer and the underlined nucleotides are the *NotI* recognition site). Glycine is an ideal amino acid for a protein linker region because it has no side chain modification and, therefore, breaks any possible secondary protein structure between the cloned ORF protein and the tag.
15. Taq DNA polymerase does not have a DNA proof-reading activity and is therefore not suitable to generate PCR-amplified DNA fragments for cloning. Taq DNA polymerase amplified PCR fragments contain between 1 and 2 random nucleotide changes per 1 kb of cloned DNA while KOD⁺ DNA polymerase-amplified DNA fragments contain only 1–2 nucleotide changes for every 50 kb of cloned DNA.

16. Make sure that the PCR machine is set to heat the lid (hot bonnet). If the lid is not heated, the liquid at the bottom of the PCR tube will evaporate and condense on the lid of the tube, leading to a failure of the PCR.
17. To visualize DNA fragments with EtBr we add 3 μL of EtBr (2 mg/mL) to the melted agarose (0.7 g agarose is melted in a microwave oven in 100 mL 1 $\times$ TAE) just before pouring the gel. After DNA gel electrophoresis at constant voltage (100 V; in 1 $\times$ TAE buffer) DNA fragments can be readily visualized on a UV transilluminator.
18. A typical 50 μL PCR reaction should produce 1–10 μg of amplified DNA. Thus, a 1 μL portion of a typical PCR should contain 10–200 ng of amplified DNA product. If the yield of amplified DNA product is lower than 10 ng/ μL , the PCR was not efficient enough and needs to be optimized. An inefficient PCR can lead to a significantly higher error rate by the DNA polymerase. KOD⁺ DNA polymerase has a narrow $[\text{Mg}^{2+}]$ optimum usually 1–1.2 mM. To identify the optimum $[\text{Mg}^{2+}]$, test a concentration range between 0.8 and 2 mM Mg^{2+} . If that does not improve the PCR yield (and quality of the PCR product), consider the possibility that either the primer design is suboptimal, the quality of the synthesized primers is poor (*see* also **Note 3**), or there are errors in the primers that were synthesized.
19. A number of other DNA purification kits are commercially available, but from our experience the QIAquick[®] PCR purification kit from Qiagen is of superior quality leading to the greatest DNA recovery (usually 80–100%) while other products often result in less than 50% recovery. Since the cloning of *CaCDRI* requires three purification steps [two column purifications and one gel purification (*see* **Note 27**)], a 50% loss of DNA at each step would result in 12.5% overall DNA recovery, compared to about 50% when using the QIAquick[®] PCR purification kit.
20. It is important to remove the wash buffer because it contains a high EtOH concentration and DNA is insoluble in EtOH. Therefore, to ensure that the column is dry, remove (with a pipetter) all residual wash buffer that is caught around the rim of the spin column that holds the DNA-binding material in place, and spin the column at maximum speed in a microfuge for another minute. This is an essential step and failure to remove wash buffer is the most common cause for bad DNA recovery.
21. The portions of the PCR products from before (1 μL of 50 μL ; Step 6 of **Section 3.2.3**) and after the column purification (1.2 μL of 60 μL ; Step 7 of **Section 3.2.4.1**)

should be identical and, therefore, allow a good estimation of the DNA recovery by direct comparison of the intensities of the EtBr-stained DNA bands of the PCR when run on the same agarose gel.

22. To digest the *CaCDRI* PCR amplicon efficiently with both restriction enzymes, *PacI* and *NotI*, digest the DNA first with *PacI* and then with *NotI*. Often it is possible to digest DNA with two different restriction enzymes in the same buffer (*see* NEB catalogue). Unfortunately, this is not so for *PacI* and *NotI*. *PacI* requires NEB buffer 1 while *NotI* requires NEB buffer 3. To change buffers use the QIAquick[®] PCR purification kit as described in **Section 3.2.4.1**.
23. This step makes sure that the *CaCDRI* PCR amplicon is fully digested with *PacI* as well as *NotI*. Most restriction enzymes are unstable at 37°C and can lose a significant amount of activity within a few hours, and it is therefore better to use smaller amounts of enzyme repeatedly, rather than a large amount only once.
24. Gel purification is essential because the very small *PacI* and *NotI* adapters, that are a side product of the digestion of the *CaCDRI* PCR amplicon with *PacI* and *NotI*, ligate 100–1,000 times more efficiently into the *PacI/NotI* cut pABC3 or pABC3-His plasmid than the much larger *PacI/NotI CaCDRI* ORF fragments. Contrary to the manufacturer's claims, experience in our laboratory has shown that the QIAquick[®] PCR purification kit does not separate efficiently small DNA molecules such as DNA primers or small *PacI* or *NotI* adapters from the larger PCR products, even though they are not visible on an agarose gel.
25. Exposing DNA to short wavelength (high-energy) UV light for as little as 1 min can cause serious damage to the DNA. Many new UV transilluminators have an option to switch between low- and high-energy UV, specifically for the purpose of isolating DNA from agarose gels while visualizing the EtBr-stained DNA band under direct UV light.
26. The larger the piece of agarose that has been cut out of the gel, the lower the DNA recovery. For good DNA recovery, the excised agarose gel fragment should not weigh more than 400 mg.
27. A number of commercially available kits are available for the gel purification of DNA fragments. Here too, based on our experience, the QIAquick[®] gel extraction kit from Qiagen leads to the best results (close to 100% recovery; *see* also **Note 19**).

28. If all the purification steps were 100% efficient, than 1.2 μL of gel purified, *PacI*/*NotI*-digested, *CaCDR1* should contain a similar amount of DNA as 1 μL of the original PCR reaction (Step 6 of **Section 3.2.3**). Running a gel to compare *CaCDR1* DNA before (1 μL) and after (1.2 μL) the digestion and gel purification steps will give a good estimation of the DNA recovery. A good procedure should recover about 40–50% of the DNA from the original PCR. However, DNA recoveries can vary depending on technical ability and can be as low as 5%.
29. Self-ligation (intramolecular ligation) between two ends of the same linear piece of DNA molecule is at least 1,000-fold more efficient than ligation between two different DNA molecules (intermolecular ligation).
30. We routinely keep plasmid stocks at a concentration of ~ 200 ng/ μL . Conveniently, this is the concentration that is usually achieved when isolating high-copy number plasmids such as pABC3 from *E. coli* transformants with the QIAprep[®] spin miniprep kit. The maximum DNA binding capacity of mini-prep columns is ~ 10 μg . Routinely, plasmid mini-preps are eluted in 50 μL of elution buffer, resulting in ~ 200 ng/ μL plasmid stocks. Plasmid stocks are stable for several years when stored at -20°C .
31. Plasmid DNA isolated from *E. coli* is mostly supercoiled. Supercoiled plasmid DNA runs about 1.5 times faster than a relaxed, circular, or linear, piece of DNA of the same number of base pairs. Old plasmid DNA preparations (>1 year) or plasmid DNA preparations that have been thawed many times tend to accumulate more and more single strand DNA nicks in one of their two DNA double strands and become more relaxed with time.
32. To confirm that the *NotI* enzyme is still active and has cut both plasmids to completion, a control digest of 2 μg uncut pABC3 with *NotI* is necessary. This is because pABC3 plasmids that have been successfully cut with both *PacI* and *NotI* enzymes appear identical to *PacI*-only digested pABC3 plasmids on a DNA agarose gel as they are of very similar size. It is not uncommon that restriction enzymes lose their activity when stored at -20°C for a long time, and this control is therefore recommended to ensure the enzyme is still fully active.
33. This step is necessary to confirm that all DNA fragments have been purified successfully, are of the correct size, and to determine their concentrations. To determine the DNA concentration compare the intensities of the EtBr-stained,

purified, DNA fragments with the known amount of one of the EtBr-stained bands in the 1 kb plus DNA ladder. The estimation of amounts of DNA from EtBr-stained DNA agarose gels is accurate enough for all molecular biological procedures described in this chapter.

34. The high PEG 6000 concentration in the 2× ligation buffer could cause the DNA (*CaCDRI* and pABC3) to precipitate at low temperature and thus dramatically reduce the ligation efficiency, so warm it to RT.
35. Set up a control ligation of plasmid only, replacing the insert with H₂O. This control ligation will help determine the success of the cloning procedure.
36. The ligation reaction is very efficient and complete within 1 h at RT.
37. The ligation reaction can be safely stored for weeks at 4°C.
38. In order to resuspend the cell pellet in a large volume of buffer (e.g., 80 mL TB) loosen the pellet first by stirring the cells with a pipette tip, then add a small amount (1–5 mL) of buffer, gently pipette cells up and down, and swirl the centrifuge tube (keep on ice) until the cells are fully resuspended, before adding the rest (75 mL) of the buffer.
39. Freshly prepared competent *E. coli* DH5α cells routinely give a transformation rate of $1\text{--}5 \times 10^5$ transformants/ng of control uncut pABC3 plasmid DNA.
40. To test the quality of the transformation always include a transformation with a known amount (e.g., 1 ng) of an uncut plasmid such as pABC3 as a positive control experiment. Transformation with 1 ng pABC3 and plating 10 μL of 1 mL recovered *E. coli* cells should give $1\text{--}5 \times 10^3$ transformants on the plate.
41. For the recovery of transformed *E. coli* cells add 20 mM glucose to 10 mL of SOC medium and pre-warm it in the 42°C water bath that is being used to transform the cells. A simple procedure is to keep 10 mL aliquots of sterile SOB medium stored at RT and add 100 μL of sterile 2 M MgCl₂/MgSO₄ stock solution (*see Section 2.2*) and 100 μL of a sterile 2 M glucose stock solution when needed.
42. To plate small volumes of cells evenly simply dilute a 1 μL or 10 μL portion of transformed cells into ~90 μL SOC medium before spreading. The remaining portion of the recovered *E. coli* cell suspension can be stored at 4°C for weeks.
43. The experiment can be considered to have failed if the background number of transformants on the control plate

(100 μ L of recovered *E. coli* cells transformed with 4 μ L of the vector only control ligation; *see* **Note 35**) is higher than 100 or if the plate containing *E. coli* that was transformed with the vector plus insert (pABC3 plus *CaCDRI*) has a similar (low) number of transformants as the background control plate. Common reasons for failure are (a) the vector or the insert has not been cut with one of the two enzymes (low or no enzyme activity); (b) the primers have a point mutation or, more commonly, a deletion (for an explanation, *see* **Note 3**), in either the *PacI* or the *NotI* site; or (c) the ratios of insert to vector are not optimal [e.g., not enough insert (<1:1 ratio of insert to vector molecules) or too much insert (>100:1 ratio of insert to vector molecules) – this may cause self-ligation of insert].

44. A 3 mL overnight culture should yield sufficient plasmid DNA to saturate a mini-prep column (10 μ g). However, the cloning of larger DNA fragments (≥ 5 kb) reduces the plasmid DNA copy number per *E. coli* cell and, therefore, can lead to a significantly reduced yield of plasmid DNA (1–5 μ g only).
45. 1 U of a DNA restriction endonuclease is defined as the amount of enzyme that is needed to digest to completion 1 μ g of DNA containing one recognition site in 1 h at the recommended temperature (usually 37°C).
46. Always make slightly more master mix than required (make a 6 $\times$ mix if 5 are required or an 11 $\times$ master mix if 10 are required). This is because some liquid is always lost during multiple pipetting steps.
47. DNA oligomer primers for sequencing only need to be of “ready desalted” quality.
48. If the sequenced plasmid has a point mutation, select another one that shows the correct restriction pattern and confirm that one by sequencing instead.
49. From experience, most sequencing errors occur in the DNA corresponding to the primers that were used to amplify the *CaCDRI* ORF. Most of these sequence variations are deletions due to errors in the chemical synthesis of the primers and are quite common (for an explanation, *see* **Note 3**). A deletion of one nucleotide in the 3' primer just downstream of the ATG start codon of the ORF would lead to a frameshift mutation and an inactive protein. These types of mutations occur at a frequency of about 5–20% and are much more common than errors derived from the KOD⁺ DNA polymerase that is used to PCR amplify the *CaCDRI* ORF (for the accuracy of KOD⁺, *see* **Note 15**).

50. Perform the digest in a final volume of 20–100 μL and use 10 U of *AscI* to digest 2 μg of the plasmid for 1 h.
51. Plasmid pABC3-CaCDRI contains two unique *AscI* restriction sites, one at each end of the transformation cassette (**Fig. 15.1A**).
52. The *S. cerevisiae* ADA strain is deleted in seven ABC transporters (**Fig. 15.1A**) and, therefore, is very sensitive to many xenobiotics, drugs, and stress conditions such as high concentrations of Li^+ cations that are used in this step of the protocol. Therefore, the yeast transformation protocol recommended by the manufacturer of the alkalization transformation kit had to be specifically modified to suit the ADA strain. The major modifications are the amount of cells used (250 mL YPAD culture instead of the recommended 50 mL), the shorter incubation (25 min) of the cells with Li^+ cations in this step, and a 60 min long heat-shock treatment at 42°C as opposed to the recommended 10 min. Using smaller cultures (50 mL versus 250 mL) and, therefore, $5\times$ less cells per 100 μL competent cells or longer than 30 min incubations with Li^+ cations dramatically reduce the transformation efficiency.
53. The transformation frequency obtained with these ADA cells is about $1\text{--}2 \times 10^4$ transformants/ μg of plasmid DNA or about 100 times lower ($2\text{--}5 \times 10^2/\mu\text{g}$) for linear DNA molecules that have to integrate into the chromosome via a double homologous cross-over event, as is the case for the transformation cassette used in this experiment. We routinely use 20 ng ($\sim 1 \mu\text{L}$ of a diluted plasmid stock) of the common yeast episomal plasmid pYES2 as a positive control to test the transformation frequency for the competent cells; however, any other yeast episomal plasmid containing a *URA3* selection marker can also be used as a positive control.
54. Transformation-competent yeast cells can be stored at -80°C long-term (at least a few weeks without significant loss of competency) as a 15–25% glycerol stock.
55. Make a small hole in the closed lid of the microfuge tube by stabbing the lid with a needle so that the lid does not pop-open when the microfuge tube is placed in the boiling water bath.
56. The boiling step ensures that the carrier DNA is fully denatured. Single-stranded carrier DNA of an average size larger than 2 kb improves the transformation rate by a factor of 100 (10). It is therefore important to ensure that the carrier DNA that is kept at 4°C is fully denatured before use. At the same time it is important not to boil the carrier DNA

too often to avoid the hydrolysis of the DNA and maintain the average size of the DNA >2 kb.

57. Although recommended by the manufacturer, histamine is not essential, has no influence on the transformation rate, and may be left out.
58. Prepare the mix in a 15 mL Falcon tube by thoroughly mixing 0.8 mL PEG solution with 0.2 mL TE/cation MIXX solution per transformation. This solution is quite viscous, and in order to make sure that the competent cells are resuspended well in that solution, add 1 mL PEG/TE/cation MIXX by pipetting the solution fast into the middle of the tube. That way the 100 μ L of competent cells float on the surface of the solution and are much easier to resuspend in the PEG/TE/cation MIXX.
59. After 10 s of centrifugation the cell pellet is not very tight, but it is important not to spin any longer to prevent disrupting the fragile cells. Remove as much of the supernatant as possible before resuspending the cells in the next step.
60. Δ AD cells are deleted in the *URA3* gene (an enzyme involved in uracil metabolism) and therefore require uracil in the medium for them to grow. Δ AD cells cannot grow on CSM-ura plates unless they have been transformed with DNA containing a wild-type *URA3* gene.
61. Yeast possesses a thick cell wall that prevents the release of DNA from the cells making PCR from intact cells difficult. PCR using bacterial colonies (e.g., *E. coli* transformants picked from LBamp plates) only requires the touch of a colony with a pipette tip to get enough cell material for a successful PCR using any commercially available heat-stable DNA polymerase.
62. Include a negative control PCR using untransformed Δ AD cells. A PCR product for both DNA oligomer primer pairs can only be amplified if the transformation cassette has properly integrated at both the 5' and 3' ends of the *PDR5* locus because PDR5up (5'-GAGCATAAAACAGAGAGG-CGATATAGG-3') and PDR5down (5'-TATGAGAAG-ACGGTTCGCCATTCGGACAG-3') have been designed so that they specifically bind to the chromosomal *PDR5* locus 40 bp either side of the expected integration sites of the ends of the transformation cassette. The homologous recombination event that is required to integrate the *CaCDRI* transformation cassette at the genomic *PDR5* locus is very efficient in *S. cerevisiae*. Thus, expect the majority of Δ AD transformants (90–100%) to be positive for both the 5' and 3' PCR reactions. The DNA

sequences of the other two DNA oligomer primers that are used to test the integration of the *CaCDR1* transformation cassette into the *PDR5* locus are *CaCDR1*-rev: 5'-TCACCACCGGAA-ACACCACGG-3' and *URA3*-for: 5'-CAAATTGCAGTACTCTGCGGGTG-3'.

63. Do not use too many cells. Generally the less the cell material used for the colony PCR the better the results. Using too many cells can inhibit the PCR. A bad yeast colony PCR usually shows a large amount of primer-dimer formation. DNA primer-dimers appear as thick bands at the bottom of EtBr-stained DNA agarose gels after analysis of the PCR by gel electrophoresis.
64. We routinely add RNase A (100 µg/mL) to the Y-PER reagent to remove all possible RNA contamination. The RNase A is added directly into the stock of the Y-PER reagent and remains active in that buffer for many months (the kit is stored at 4°C).
65. Take no more than 1–2 µL of cell material by stabbing a single yeast colony.
66. DNA Releasing Reagent A contains SDS that precipitates when kept at 4°C. Make sure that SDS is dissolved at RT before use.

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References

1. Buckley, M. (2008) *The fungal kingdom – diverse and essential roles in earth's ecosystem. A report based on a colloquium held November 2–4, 2007*. American Academy of Microbiology, Washington, DC.
2. Cannon, R. D., Holmes, A. R., Mason, A. B., and Monk, B. C. (1995) Oral *Candida*: clearance, colonization, or candidiasis? *J. Dent. Res.* **74**, 1152–1161.
3. Kelly, S. L., Arnoldi, A., and Kelly, D. E. (1993) Molecular genetic analysis of azole antifungal mode of action. *Biochem. Soc. Trans.* **21**, 1034–1038.
4. Sanglard, D., and Bille, J. (2002) Current understanding of the modes of action of and resistance mechanisms to conventional and emerging antifungal agents for treatment of *Candida* infections, in *Candida and Candidiasis* (Calderone, R. A., Ed.). ASM Press, Washington, DC, pp. 349–383.
5. White, T. C., Marr, K. A., and Bowden, R. A. (1998) Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11**, 382–402.

6. Nakamura, K., Niimi, M., Niimi, K., Holmes, A. R., Yates, J. E., Decottignies, A., Monk, B. C., Goffeau, A., and Cannon, R. D. (2001) Functional expression of *Candida albicans* drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. *Antimicrob. Agents Chemother.* **45**, 3366–3374.
7. Lamping, E., Ranchod, A., Nakamura, K., Tyndall, J. D., Niimi, K., Holmes, A. R., Niimi, M., and Cannon, R. D. (2009) Abc1p is a multidrug efflux transporter that tips the balance in favor of innate azole resistance in *Candida krusei*. *Antimicrob. Agents Chemother.* **53**, 354–369.
8. Lamping, E., Monk, B. C., Niimi, K., Holmes, A. R., Tsao, S., Tanabe, K., Niimi, M., Uehara, Y., and Cannon, R. D. (2007) Characterization of three classes of membrane proteins involved in fungal azole resistance by functional hyperexpression in *Saccharomyces cerevisiae*. *Eukaryot. Cell*, **6**, 1150–1165.
9. Inoue, H., Nojima, H., and Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, **96**, 23–28.
10. Schiestl, R. H., and Gietz, R. D. (1989) High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16**, 339–346.

Section III

Cells and Tissues

Chapter 16

Explant Culture of Embryonic Craniofacial Tissues: Analyzing Effects of Signaling Molecules on Gene Expression

Katja Närhi and Irma Thesleff

Abstract

The *in vitro* culture of embryonic tissue explants allows the continuous monitoring of growth and morphogenesis at specific embryonic stages. The functions of soluble regulatory molecules can be examined by adding them into culture medium or by introducing them with beads to specific locations in the tissue. Gene expression analysis using *in situ* hybridization, quantitative PCR, and reporter constructs can be combined with organ culture to examine the functions of the regulatory molecules.

Key words: Mouse, morphogenesis, organ culture, tooth, whisker, palate, calvarial bone, *in situ* hybridization, real-time quantitative PCR.

1. Introduction

The development of embryonic organs is characterized by dynamic morphogenetic events such as budding, branching, and tissue fusions which are accompanied by growth, migration, and differentiation of cells. Central mechanisms regulating these developmental processes are interactions between cells and tissues. In the case of ectodermal organs like teeth and hairs, interactions between the epithelium and underlying mesenchyme are of particular importance. The formation of the epithelial placode and the subsequent folding of the epithelium in an organ-specific manner are regulated by the reciprocal and sequential interactions between the epithelium and mesenchyme. Cell and tissue interactions are mediated by soluble signaling molecules which are

conserved in evolution and regulate the development of all organs. The most important and widely used signals belong to four families: bone morphogenetic protein (BMP), hedgehog (HH), fibroblast growth factor (FGF), and the Wnt family. The signals are integrated into complex regulatory networks in which specific inhibitors of signals also play important roles in fine-tuning signaling. A good example of an organ where the functions of signaling networks have been elucidated in detail is the mouse tooth (1).

To follow the morphogenesis of developing organs, the tissues can be transplanted *in vivo* or cultured as explants *in vitro*. Organ culture is not suitable for long-term follow-up and is not offering physiological environment which are the advantages of the transplantation method. However, organ culture techniques are superior in many other aspects. These techniques allow continuous monitoring; they provide reproducible conditions, known medium composition, and, importantly, tissues can be manipulated by multiple controlled ways. Over the years, several types of organ culture systems have been used for the examination of embryonic morphogenesis. A technique employing a platform of perforated metal gauze is called Trowell-type organ culture (2) and it has been applied to study the morphogenesis of numerous different organs (3–8). In this technique tissue explants are cultured at the medium/gas interface on thin membrane filters supported by a metal grid (Fig. 16.1). We have applied the Trowell

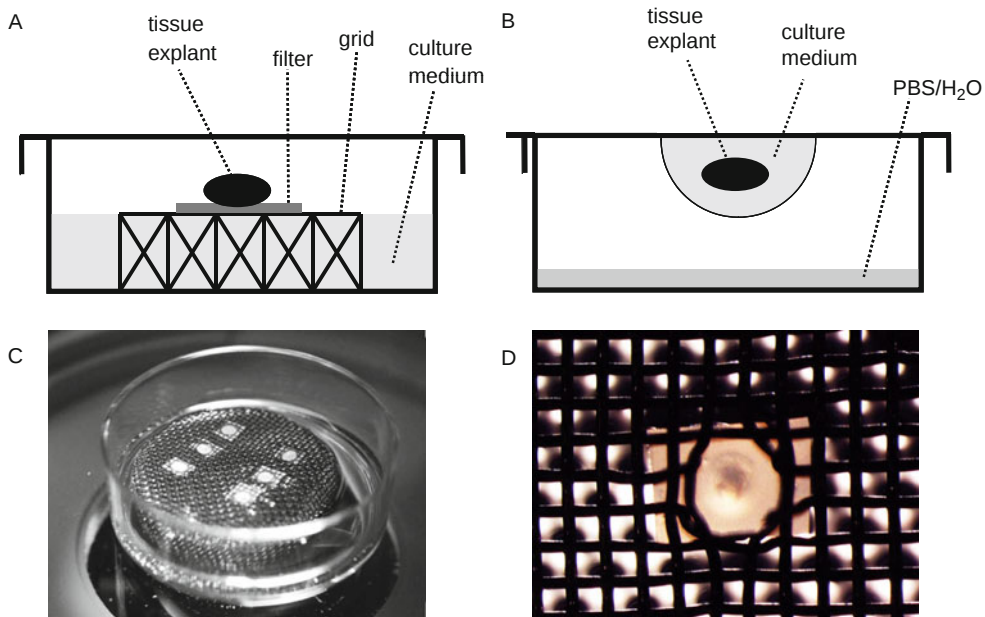


Fig. 16.1. Schematic representations of **A** Trowell-type organ culture and **B** Hanging-drop technique. **C** The Trowell-type organ culture dish where the metal grid supports six pieces of filters placed on the holes punched in the grid. **D** Close-up of one cultured explant lying on a filter in the Trowell-type culture dish (Picture **C** courtesy of Otso Häärä).

technique modified by Saxén (9) to elucidate the mechanisms of tissue interactions during embryonic tooth development (10–17) and also for studies on the fusion of palatal shelves, osteogenesis of calvarial bones, and suture formation, as well as the formation of whiskers (8, 18–20).

Recombinant signaling molecules or their inhibitors can be applied either in the culture medium or locally by beads to examine their regulatory functions. Gene expression can be examined during culture by using tissue from transgenic reporter mice expressing fluorescent markers. Alternatively, gene expression can be localized in fixed tissues by in situ hybridization analysis either from the whole explants or from tissue sections. This method is based on labeled (radioactive or non-radioactive) RNA (ribo-probe) which binds to corresponding mRNA sequences produced by the tissue (21).

In addition to the Trowell-type organ culture we have recently used the so called hanging-drop culture method (Fig. 16.1) and combined it with real-time quantitative PCR (qPCR) to accurately quantify gene expression changes (22, 23). The hanging-drop technique requires only a tiny volume of culture medium, allowing smaller consumption of expensive signaling molecules, and the harvesting of the tissues from the drops is effortless. Exposing tissue explants over very short periods of time to signaling molecules allows the detection of their direct targets by real-time qPCR on complementary DNA produced from the tissue. In this chapter we describe two organ culture techniques: the Trowell and the hanging-drop methods, which we have used for culturing teeth, palate, whiskers, and calvarial bones. We also present the techniques for tissue processing for gene expression analysis by in situ hybridization and describe the quantitative RT-PCR method.

2. Materials

2.1. Solutions and Culture Media

All solutions should be sterile.

1. Phosphate-buffered saline (PBS), pH 7.4. 10× PBS stock solution: 1.37 M NaCl, 27 mM KCl, 79 mM Na₂HPO₄ × 2H₂O, 15 mM KH₂PO₄. Adjust pH with HCl if necessary. Prepare 1× PBS working solution by diluting one part of 10× stock solution with nine parts of distilled water. Autoclave the working solution and store at room temperature.
2. Dulbecco's phosphate-buffered saline, modified (D-PBS) (HyClone/PerBio, Utah, USA). Store at room temperature.
3. Culture medium: Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, Steinheim, DE; store at 4°C)

supplemented with 1% (v/v) glutaMAX-1 (Gibco/Invitrogen, Paisley, UK; store in single-use aliquots at -20°C), 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone/Thermo Scientific, South Logan, Utah, US, research grade EU approved; freeze in single-use aliquots at -20°C), and 0.2% (v/v) PS (Gibco/Invitrogen, Paisley, UK; penicillin 10,000 IU/mL, streptomycin 10,000 $\mu\text{g/mL}$; freeze in single-use aliquots at -20°C). PS is stable in medium for 4 weeks. Store culture medium at 4°C . Usually 50 mL of culture medium is enough for one experiment.

4. F-12 (Ham's nutrient mixture, Gibco/Invitrogen, Paisley, UK). Store at 4°C . Use as 1:1 mixture in culture medium (*see Note 1*).
5. Ascorbic acid (Merck, Darmstadt, DE, pro analysi): Prepare 10 mg/mL stock solution in distilled water and freeze in single-use aliquots at -20°C . Use 100–150 $\mu\text{g/mL}$ in culture medium (*see Note 1*).

2.2. Dissection and Culture

All glassware and metal instruments should be sterile. We use autoclaved glassware. For sterilization of forceps and scissors we use Steri 250 glass bead sterilizer (Simon Keller Ltd. Burgdorf, CH).

1. Dissection of tissues: 10-cm diameter plastic bacteriological Petri dishes (Bibby Sterilin Ltd., Stone, Staffs, UK) and 10-cm diameter glass Petri dishes, small scissors (Instrumed 96, DE), forceps (Medicon, DE), watchmaker forceps (Dumont, CH), and disposable 20- and 26-gauge needles (Terumo, Neolus, Leuven, BE) attached to 1-mL plastic syringes (Euromedis) (*see Note 2*).
2. Culture dishes: 35 mm/10 mm plastic Petri dishes (bacteriological or cell culture dishes, Greiner Bio-One, DE).
3. Metal grids: Prepare from stainless-steel mesh (corrosion resistant, size of mesh 0.7 mm) by cutting approximately 30 mm diameter disk and bending the edges to give 3 mm height (the height of the metal grids can be altered affecting the amount of culture medium needed). Use nails to make holes in the grid to allow the analysis and photography of the explants (**Figs. 16.1** and **16.2**) There are commercially available organ culture dishes featuring a central well in which a metal grid (even without bent edges) can be placed (Falcon, Becton Dickinson Ltd., Oxford, UK).
4. Filters: 25-mm diameter Nuclepore[®] Polycarbonate Track-Etch Membranes (Whatman, Schleicher & Schuell, DE). The pore size routinely used is 0.1 μm (*see Note 3*). The filters are stored in 70% ethanol at room temperature.

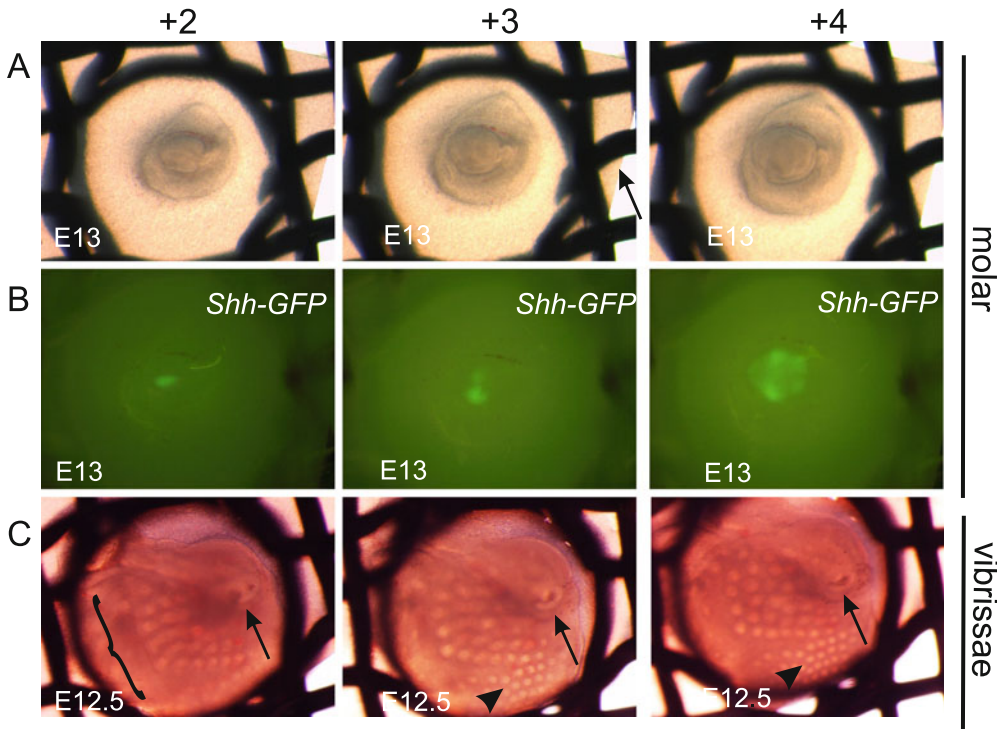


Fig. 16.2. Use of Trowell-type culture to follow the morphogenesis of a molar tooth and the initiation of whiskers. **A, B** The bud-staged molar was dissected from the mandible of an E13 transgenic mouse embryo expressing GFP in the *Shh* locus (30) and placed on a piece of Nuclepore filter (the arrow points to the edge of the filter) covering the hole of a metal grid. The occlusal side is *up*, the mesial side on *left*, and the distal side on *right*. The explant was photographed using **A** light and **B** fluorescent microscopy. **B** After 2 days of culture (+2), GFP expression is localized to a spot in the center of the molar indicating the formation of the primary enamel knot and development to cap stage. The following day (+3), new GFP expressing spots indicate the formation of secondary enamel knots for protoconid and metaconid and progress to early bell stage, and on the last culture day (+4) three additional secondary enamel knots for anteroconid, hypoconid, and entoconid are detected. **C** E12.5 vibrissa pad was dissected, cultured, and photographed daily using stereomicroscope. After 2 days of culture (+2) all five whisker rows are seen (*brace*). During days +3 and +4, hair placodes (*arrow head*) are detected under the whisker rows. *Arrow* points to the developing nostril (pictures **A** and **B** courtesy of Enni Penttälä).

5. Protein-releasing beads: 17–150 μm diameter Affi-Gel Blue agarose beads (Bio-Rad Laboratories, Hercules, CA) or heparin-coated acrylic beads (Sigma, St. Louis, MO) are divided into aliquots and stored at 4°C.
6. Glass Pasteur pipettes are used for transferring beads and tissue explants. Before transferring beads, pipettes are drawn by heating to adjust the mouth of the pipette to the size of beads. Ideally, the diameter should be the minimal to allow free passage of the beads.
7. Stereomicroscope (e.g., Olympus SZX9, JP) attached to a camera (e.g., Olympus DP12, JP).
8. Pre-fixation after culture: Methanol (Sigma-Aldrich, Steinheim, DE), store at 4°C.

9. Fixation: 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) in PBS, freeze in aliquots (15 mL) at -20°C . It is recommended to use fresh PFA (after melting, store at 4°C and use within 2 days).

2.3. Complementary DNA (cDNA) Synthesis

1. RNA isolation: RNeasy Mini Kit (Qiagen, Hilden, DE), β -mercaptoethanol (Sigma-Aldrich, Steinheim, DE, for Molecular Biology). Store at room temperature.
2. cDNA synthesis: Random Primers (Promega, Madison, WI; 500 $\mu\text{g}/\text{mL}$), RNase inhibitor RNasin[®] (Promega, Madison, WI; 40 U/ μL), dNTP mix (Finnzymes, Espoo, FI; 10 mM), Superscript[™] II Reverse Transcriptase (Invitrogen, Carlsbad, CA; 200 U/ μL ; this kit includes the 5 $\times$ first-strand buffer and 0.1 M DTT, as well). Store all reagents at -20°C .

2.4. Real-Time Quantitative PCR (RT-qPCR)

1. DyNAmo[™]Flash SYBR Green qPCR Kit (Finnzymes, Espoo, FI). Store at -20°C .
2. Primers for sample and control cDNA (design with Primer 3 software and order from Sigma-Aldrich), store at -20°C .
3. LightCycler 480 Multiwell Plate 96 or 384 with sealing foils (Roche, Mannheim, DE). Store at room temperature.
4. For running qPCR: LightCycler 480 machine (Roche, DE).
5. For analysis of qPCR data: Lightcycler 480 software (Roche, DE).

3. Methods

The preparation of materials and dissection of tissues is carried out in a laminar flow hood. The dissection microscope should be placed in the hood, as well.

3.1. Treatment of Beads

1. Pipette agarose beads or heparin-coated acrylic beads to PBS in a glass Petri dish. Count 100–200 beads under the microscope and transfer to a non-stick Eppendorf tube with mouth-controlled glass capillary pipette. Spin down the beads and remove PBS.
2. Add recombinant proteins in a small volume (10–50 μL) of 0.1% bovine serum albumin (BSA) in PBS. In general, high concentrations of proteins are used. For instance, we use 20–25 ng/ μL of FGF4 (R&D Systems, Minneapolis, MN) and 100 ng/ μL of BMP4 (R&D Systems, Minneapolis, MN). An equal amount of 0.1% BSA in PBS is pipetted to

control beads. Incubate for 30 min at 37°C and store at 4°C. The beads can be used at least for 14 days (depending on the stability of the protein).

3.2. Preparation of Tissue Culture Dishes

1. Take one sheet of Nuclepore filter from ethanol and rinse three times in PBS in a plastic 10-cm diameter Petri dish.
2. Cut the filter in pieces (3–5 mm² depending on the size of tissue), using small scissors and watchmaker forceps and leave in PBS. Filter pieces can be stored in PBS for several days at 4°C.
3. Place metal grids in 35-mm diameter plastic Petri dishes. Add 1–3 mL culture medium (DMEM supplemented with FBS, PS, and glutaMAX-1, *see Note 1*) by pipetting through the grid. Avoid air bubbles. The surface of the medium should be flush with the plane of the grid. Excess medium results in floating of the filters and tissues.

3.3. Dissection of Tissues

1. Place the mouse uterus (E11–E18) in a 10-cm diameter plastic Petri dish containing D-PBS and cut open the uterine wall using small scissors and forceps. Under stereomicroscope remove the embryos from fetal membranes and transfer them to a fresh plastic Petri dish with D-PBS. Cut off the heads using disposable needles (or with scissors when dissecting older embryos) and transfer the heads to a 10-cm diameter glass Petri dish containing D-PBS.
2. Dissect the tissue piece of interest using needles: mandible, calvarial bones, vibrissae pads, or tooth buds (*see Notes 4 and 5*). If tissues are cultured using hanging-drop technique continue as described below (*see Section 3.6*). For hanging-drop technique it is essential to remove all the extra tissue surrounding the tissue of interest to avoid skewed data in real-time qPCR.
3. Pipette warm (37°C) culture medium (supplemented with FBS, PS, and glutaMAX-1) to a 35-mm plastic Petri dish with a grid. Transfer the dissected tissue pieces on the metal gauze by lifting with a filter piece and watchmaker forceps. Alternatively, the explants can be transferred by the capillary force between the tips of watchmaker forceps or with the Pasteur pipette and placed on filters lying on the metal grid.
4. Add signaling molecules (*see Note 6*) either directly to culture medium or introduce them with beads soaked in a high concentration of molecules. Under the stereomicroscope, transfer the beads one at a time to the tissues. Depending on the experiment and tissue, 1–5 beads can be placed on one

explant. Examples of BMP4- and BMP2-bead experiments are shown in **Fig. 16.3** (*see Note 7*).

3.4. Culture and Fixation

1. Culture the tissues in a standard incubator at 37°C, in an atmosphere of 5% CO₂ in air and 90–95% humidity. The culture medium should usually be changed every other day (*see Note 8*).
2. Photograph the explants, e.g., daily with a camera attached to the stereomicroscope (*see Note 9*).
3. Remove the culture medium by sucking and pipette ice-cold methanol (pre-fixation) gently on the tissues to avoid detachment of tissues from the filters. Leave for 5 min and transfer filters by watchmaker forceps to Eppendorf tubes to fix the explants in 4% paraformaldehyde (PFA) for 10–24 h at 4°C. Continue with gene expression analysis.

3.5. In Situ Hybridization (ISH)

1. To perform ISH on tissue sections, process tissue explants to paraffin using standard protocols. Cut serial sections of paraffin-embedded tissue explants and process for ISH to analyze the expression of genes of interest by using ³⁵S-UTP- or digoxigenin-labeled riboprobes. ISH is performed according to a protocol described in (21) with modifications (12, 24). An example of radioactive ISH is shown in **Fig. 16.3**.
2. For whole-mount ISH (with digoxigenin-labeled riboprobes) the tissue explants are rinsed in PBS and dehydrated in methanol series 25, 50, 75, and 100% (dilutions in PBS; each step 5–10 min at room temperature) after fixation. Wash with 100% methanol twice and store samples in 100% methanol at –20°C until use (can be stored for several months). Process for ISH according to instructions described in (13, 25, 26) (*see Note 10*).

Fig. 16.3. (continued) Examples of bead experiments combined with in situ hybridization (ISH) analysis. All explants were cultured for 24 h before fixation for gene expression analysis. **A–C** Inhibition of BMP2-induced *Msx2* expression by SOSTDC1 (Ectodin) in E13 tooth buds (28). **A** BMP2-releasing bead has induced *Msx2* in the immediate surroundings of the bead. **B** A SOSTDC1 (15 ng/mL)-releasing bead placed next to the BMP2 bead has markedly reduced the expression of *Msx2* and **C** several SOSTDC1 beads have completely inhibited the inductive effect of BMP2. **D** BMP4-releasing beads on E12 palatal mesenchyme. Whole-mount ISH shows induction of *Msx2* expression in the immediate surroundings of the bead. **E, F** *Id1* expression is induced in explants of E15 calvarial mesenchyme between the approximating parietal bones (p) around **E** BMP2- and **F** BMP4-releasing beads (arrow). **E** Whole-mount ISH, **F** radioactive ISH on histological section. **G, H** E16 mandibular incisors cultured with **G** a control bead soaked in BSA and **H** a BMP4-releasing bead. Whole-mount ISH indicates stronger and more enlarged *Ameloblastin* expression in the explant exposed to BMP4 compared to BSA control. The apical side of the incisor is on the *left*, the proximal side on the *right*, and the lingual side on the *top* (Pictures **A–C** courtesy of Johanna Laurikkala, **D–F** courtesy of David Rice, and **G** and **H** courtesy of Marika Suomalainen).

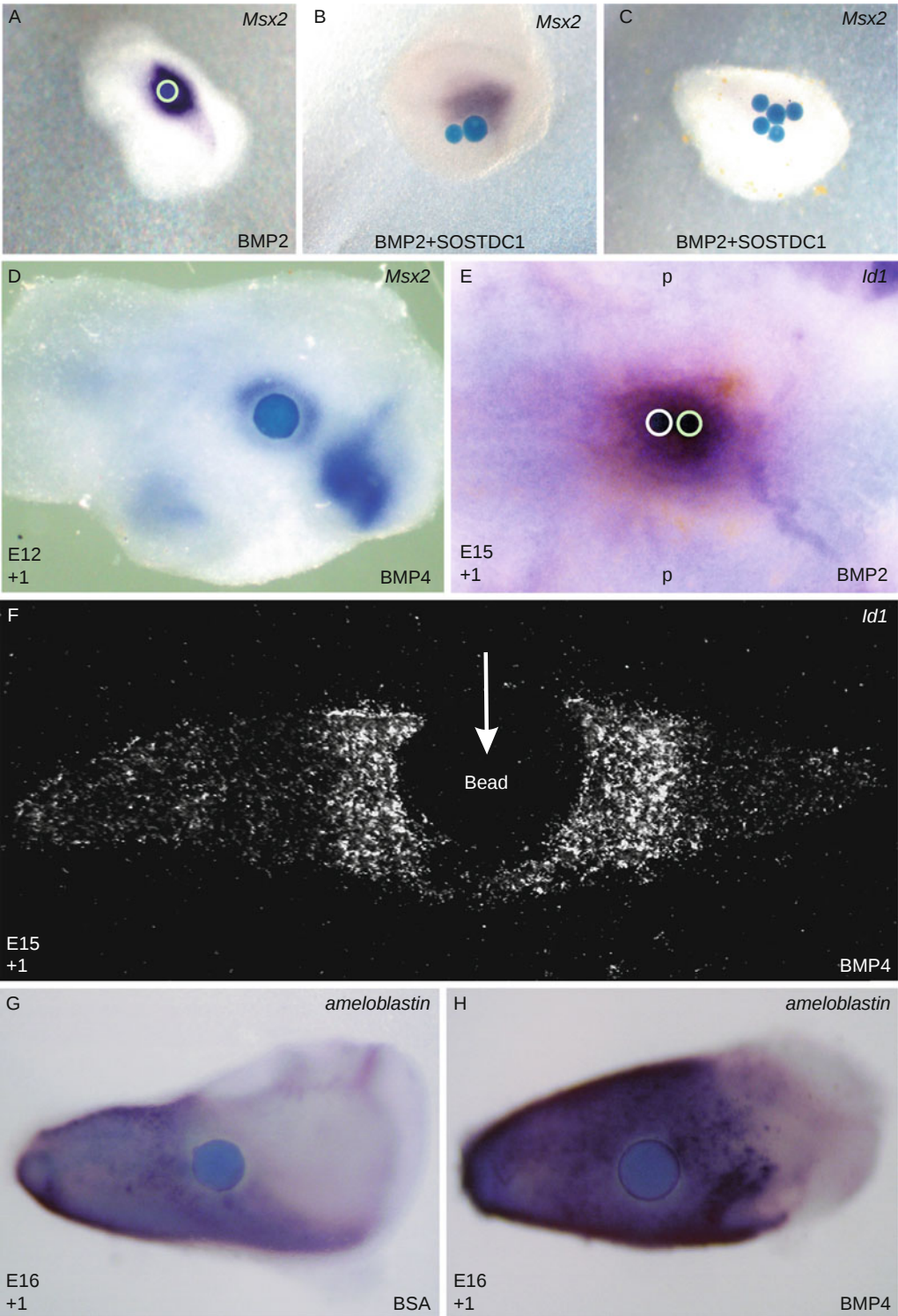


Fig. 16.3. (continued)

Examples of whole-mount ISH staining are shown in Fig. 16.3.

3.6. Hanging-Drop Culture

1. Pipette 30–40 μL drops of warm culture medium on the lid of a 35-mm plastic Petri dish. Different signaling molecules or other molecules are added to culture medium, e.g., BMP4 (0.53 ng/ μL), SHH (2 ng/ μL), or FGF4 (0.12 ng/ μL). The control culture medium is supplemented with the solvent used to dissolve the protein of interest to eliminate any effects caused by solvent or dilution of the medium.
2. Transfer the dissected tissue samples carefully to the drops using the capillary force between watchmaker forceps.
3. Turn the lid quickly and place on top of the Petri dish containing 1–2 mL of sterile liquid (PBS or distilled water) in the bottom to prevent evaporation from the hanging drop.
4. Culture as described above (*see* Section 3.4). Culture time is usually not more than 24 h.

3.7. RNA Isolation and cDNA Synthesis

1. After culture in hanging drops, lids are turned upside down to allow the collection of tissue samples under stereomicroscope with small forceps into Eppendorf tubes (*see* Note 11).
2. Lyse the tissues with 350 μL of Qiagen lysis buffer supplemented with 1% β -mercaptoethanol and isolate RNA immediately according to manufacturer's (Qiagen) instructions. Store RNA samples at -80°C and avoid repeated thawing (use aliquots!).
3. Quantify the total RNA with UV spectroscopy, absorbance at 260 nm (e.g., Nanodrop spectrophotometer requires only 1 μL of sample and gives reliable results). Quality may be checked, as well, if preferred.
4. Synthesize complementary DNA (cDNA) from total RNA according to instructions specified by manufacturer (Invitrogen). Transcribe 100–1,000 ng of total RNA with 500 ng of random primers and 100 U of Superscript II. Dilute cDNA samples with distilled water to get final volume of 100 μL and freeze in aliquots at -20°C .

3.8. Real-Time Quantitative PCR (qPCR)

1. Prepare standard series (*see* Note 12) and $10\times$ (e.g., 1–5 μM) primer mix of forward and reverse primers for each gene to be studied.
2. Prepare master mix: 2 μL $10\times$ primer mix, 3 μL H_2O , 10 μL $2\times$ LightCycler 480 SYBR Green I Master. This is for one

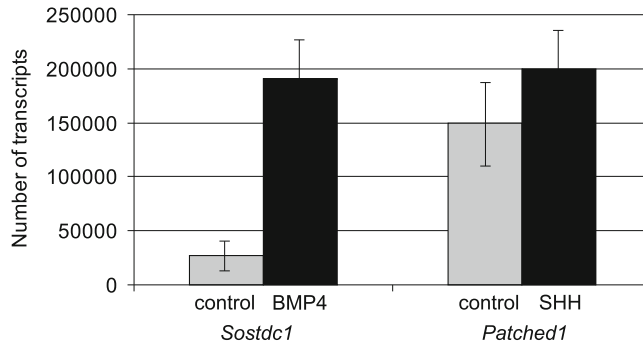


Fig. 16.4. Gene expression analysis of E13.5 dental epithelia by real-time qPCR after 3 h hanging-drop culture with recombinant BMP4 and SHH. For each treatment three replicates which all included three tooth explants were prepared. *Sostdc1* (Ectodin, Wise) is induced by BMP4 and *patched-1* by SHH. Expression is shown as number of transcripts.

reaction, so, multiply the volumes of each reagent by number of your samples (sample cDNA and standards). Also, note that you have to prepare a master mix for each gene to be studied. Mix well the prepared master mixes and keep them on ice and protected from light as they contain the SYBRGreen fluorophore.

3. Pipette 5 μ L of cDNA or standard into the wells.
4. Pipette 15 μ L of master mix into each well of the plate. The final volume is 20 μ L in each well.
5. Seal the plate with a transparent adhesive foil and use the default PCR conditions for Lightcycler 480.
6. Normalize data against *ranbp1* (tooth, mandible) or keratin 14 (skin) and analyze with Lightcycler 480 software. Gene expression is quantified by comparing the sample data against a dilution series of PCR products (*see Note 12*) of the gene of interest. An example of real-time qPCR data is shown in **Fig. 16.4**.
7. All PCR products can be separated on a 2% agarose gel using electrophoresis to check for the correct size of the PCR product and to eliminate the possibility of primer dimers.

4. Notes

1. Depending on the tissue, the composition of the optimal culture medium varies. The culture medium in this protocol is suitable for most tissues at early stages of

development, but during more advanced stages, different tissues may have special requirements. For cultures of whole tooth buds, we use culture medium composed of DMEM and F12. For more advanced stages of tooth or calvarial bone development, ascorbic acid is added to allow deposition of collagen (8, 11).

2. During dissection, it is recommended to use disposable needles instead of other instruments, such as scalpels or iris knives, because needles need no sharpening or sterilization. The size of the needles can be chosen. In addition, the syringes do not need to be changed every time as they do not have to be absolute sterile. To preserve the tissue structure, dissecting should be done by determined cuts avoiding stretching and tearing of tissue. Glass Petri dishes are superior to plastic dishes because needles easily scrape and loosen pieces from the plastic surface.
3. As supporting material, lens paper may be used for large tissue pieces. Nuclepore filters with different pore sizes (0.05–8 μm) may also be used. The maximum thickness of filters is approximately 10 μm which allows good diffusion of the medium to the tissue. Small pores (0.05–0.2 μm) allow better examination of the explants in the stereomicroscope using transmitted light, but the tissues tend to detach from these filters more readily during treatments after culture. Therefore, larger pore size (up to 0.6 μm) may be preferable, depending on the experiment.
4. The preparation and dissection of tissues should be done as quickly as possible to promote survival of the tissues. Embryos waiting to be dissected should be kept on ice in a Petri dish with D-PBS and only one uterus at a time should be prepared. The dissected tissues should be transferred to the culture dishes and the incubator within 2–3 h.
5. To study the reciprocal interactions between epithelium and mesenchyme the interacting tissues need to be enzymatically separated from each other. Various manipulations can be performed after which their advancing development is followed. Separation of epithelium and mesenchyme is described in (27).
6. Signaling molecules include proteins, growth factors, and their antagonists (28), as well as other molecules for example retinoic acid (14).
7. The experimental and control beads can be placed on opposite halves of the jaw. Antagonistic and synergistic functions of different regulatory molecules can be examined by placing beads releasing different signals or a signal and its putative antagonist near each other on the same explant (Fig. 16.3).

8. Although organs are usually cultured 1–2 weeks maximum, it is possible to culture tooth buds even for 3–6 weeks and examine the formation of roots (29).
9. Reporter mouse lines carrying green fluorescent protein (GFP)-labeled reporter constructs are useful if one wishes to follow the development of specific structure or gene expression during advancing development in vitro. GFP expression is detected by fluorescent stereomicroscope. For example, a mouse line expressing GFP in the *Shh* locus (30) is useful for tooth development studies because it allows the visualization of the dynamics of the formation of enamel knot signaling centers which express *Shh* locally (31, 32). An example of culture of *Shh*-GFP tooth buds is shown in Fig. 16.2.
10. After whole-mount ISH analysis it is possible to analyze the gross morphology of the explants (e.g., to differentiate between epithelial vs. mesenchymal staining) from vibratome sections. For this purpose explants are fixed for 24 h in 4% PFA, rinsed in PBS, embedded in gelatin/albumin, and cut to thick sections by vibratome (30–200 μm) (17, 20, 26).
11. In hanging drops, several tissue pieces can be placed in one drop depending on the size of the tissue. For instance, three to four E13 tooth germs or E11 mandibles fit well into one drop.
12. We use standard series of 10^6 , 10^5 , 10^4 , and 10^3 amplicons in 5 μL . It has to be prepared for all the genes to be studied. To calculate the number of molecules of a PCR amplicon you need to know the length (bp) and the concentration (g/ μL) of the amplicon. First, calculate the molecular weight (length $\times$ 650 g/mol) of one amplicon. Second, calculate the amount of amplicons in 1 L (concentration g/ μL $\times$ 10^6 μL) and then divide the product with the molecular weight to resolve the concentration of amplicon as mol/L. By using Avogadro's number (6.022×10^{23}), calculate the number of molecules in a liter and further in a microliter.

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References

1. Tummers, M., and Thesleff, I. (2009) The importance of signal pathway modulation in all aspects of tooth development. *J. Exp. Zool. (Mol Dev Evol)*. **312B**, 309–319.
2. Trowell, O. A. (1959) The culture of mature organs in a synthetic medium. *Exp. Cell Res.* **16**, 118–147.
3. Grobstein, C. (1953) Inductive epithelio-mesenchymal interaction in cultured organ rudiments of the mouse. *Science*. **118**, 52–55.
4. Saxén, I. (1973) Effects of hydrocortisone on the development in vitro of the secondary palate in two inbred strains of mice. *Arch. Oral Biol.* **18**, 1469–1479.
5. Saxén, L., Lehtonen, E., Karkinen-Jääskeläinen, M., Nordling, S., and Wartiovaara, J. (1976) Morphogenetic tissue interactions: mediation by transmissible signal substances or through cell contacts? *Nature*. **259**, 662–663.
6. Nogawa, H., and Takahashi, Y. (1991) Substitution for mesenchyme by basement-membrane-like substratum and epidermal growth factor in inducing branching morphogenesis of mouse salivary epithelium. *Development*. **112**, 855–861.
7. Nogawa, H., and Ito, T. (1995) Branching morphogenesis of embryonic mouse lung epithelium in mesenchyme-free culture. *Development*. **121**, 1015–1022.
8. Kim, H. -J., Rice, D. P. C., Kettunen, P. J., and Thesleff, I. (1998) FGF-, BMP- and Shh-mediated signaling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. *Development*. **125**, 1241–1251.
9. Saxén, L. (1966) The effect of tetracycline on osteogenesis in vitro. *J. Exp. Zool.* **162**, 269–294.
10. Thesleff, I., Lehtonen, E., Wartiovaara, J., and Saxén, L. (1977) Interference of tooth differentiation with interposed filters. *Dev. Biol.* **58**, 197–203.
11. Partanen, A. M., Eklom, P., and Thesleff, I. (1985) Epidermal growth factor inhibits tooth morphogenesis and differentiation. *Dev. Biol.* **111**, 84–94.
12. Vainio, S., Karavanova, I., Jowett, A., and Thesleff, I. (1993) Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell*. **75**, 45–58.
13. Jernvall, J., Aberg, T., Kettunen, P., Keranen, S., and Thesleff, I. (1998) The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. *Development*. **125**, 161–169.
14. Mitsiadis, T., Muramatsu, T., Muramatsu, H., and Thesleff, I. (1995) Midkine (MK), a heparin-binding growth/differentiation factor, is regulated by retinoic acid and epithelial-mesenchymal interactions in the developing mouse tooth, and affects cell proliferation and morphogenesis. *J. Cell. Biol.* **129**, 267–281.
15. Laurikkala, J., Mikkola, M., Mustonen, T., Åberg, T., Koppinen, P., Pispä, J., Nieminen, P., Galceran, J., Grosschedl, R., and Thesleff, I. (2001) TNF signaling via the ligand-receptor pair ectodysplasin and edar controls the function of epithelial signaling centers and is regulated by Wnt and activin during tooth organogenesis. *Dev. Biol.* **229**, 443–455.
16. Wang, X. -P., Suomalainen, M., Felszeghy, S., Zelarayan, L. C., Alonso, M. T., Plikus, M. V., Maas, R. L., Chuong, C. M., Schimmang, T., and Thesleff, I. (2007) An integrated gene regulatory network controls stem cell proliferation in teeth. *PLoS Biol.* **5**, 1324–1333.
17. Järvinen, E., Salaar-Ciudad, I., Birchmeier, W., Taketo, M. T., Jernvall, J., and Thesleff, I. (2006) Continuous tooth regeneration in mouse is induced by activated epithelial Wnt/ β -catenin signaling. *Proc. Natl. Acad. Sci. USA*. **103**, 18627–18632.
18. Rice, D., Åberg, T., Chan, Y. -S., Kettunen, P., Pakarinen, L., Maxson, R. E., and Thesleff, I. (2000) Integration of FGF and TWIST in calvarial bone and suture development. *Development*. **127**, 1845–1855.
19. Rice, R., Spencer-Dene, B., Connor, E., Gritli-Linde, A., McMahon, A. P., Dickson, C., Thesleff, I., and Rice, D. (2004) Disruption of Fgf 10/Fgf2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J. Clin. Invest.* **113**, 1692–1700.
20. Närhi, K., Järvinen, E., Birchmeier, W., Taketo, M. T., Mikkola, M. L., and Thesleff, I. (2008) Sustained epithelial β -catenin activity induces precocious hair development but disrupts hair follicle down-growth and hair shaft formation. *Development*. **135**, 1019–1028.
21. Wilkinson, D. G., and Green, J. (1990) In situ hybridization and the three-dimensional reconstruction of serial sections, in *Postimplantation mammalian embryos: a practical approach* (Copp, A. J., and Cockcroft, D. L., Eds.). IRL Press, Oxford, pp. 155–171.

22. James, M. J., Järvinen, E., Wang, X. -P., and Thesleff, I. (2006) Different roles of *runx2* during early neural crest-derived bone and tooth development. *J. Bone Miner. Res.* **21**, 1034–1044.
23. Fliniaux, I., Mikkola, M. L., Lefebvre, S., and Thesleff, I. (2008) Identification of *dkk4* as a target of Eda-A1/Edar pathway reveals an unexpected role of ectodysplasin as inhibitor of Wnt signalling in ectodermal placodes. *Dev. Biol.* **320**, 60–71.
24. Vaahtokari, A., Vainio, S., and Thesleff, I. (1991) Associations between transforming growth factor β 1 RNA expression and epithelial-mesenchymal interactions during tooth morphogenesis. *Development.* **113**, 985–994.
25. Kettunen, P., and Thesleff, I. (1998) Expression and function of FGFs-4, -8, and -9 suggest functional redundancy and repetitive use as epithelial signals during tooth morphogenesis. *Dev. Dyn.* **211**, 256–268.
26. Mustonen, T., Tümmers, M., Mikami, T., Itoh, N., Zhang, N., Gridley, T., and Thesleff, I. (2002) Lunatic fringe, FGF, and BMP regulate the Notch pathway during epithelial morphogenesis of teeth. *Dev. Biol.* **248**, 281–293.
27. Sahlberg, C., Mustonen, T., and Thesleff, I. (2002) Explant cultures of embryonic epithelium: analysis of mesenchymal signals. *Methods Mol. Biol.* **188**, 373–382.
28. Laurikkala, J., Kassai, Y., Pakkasjärvi, L., Thesleff, I., and Itoh, N. (2003) Identification of a secreted BMP antagonist, ectodin, interacting BMP, FGF, and SHH signals from the tooth enamel knot. *Dev. Biol.* **264**, 91–105.
29. Tümmers, M., Yamashiro, T., and Thesleff, I. (2007) Modulation of epithelial cell fate of the root in vitro. *J. Dent. Res.* **86**, 1063–1067.
30. Harfe, B. D., Scherz, P. J., Nissim, S., Tian, H., McMahon, A. P., and Tabin, C. J. (2004) Evidence for an expansion-based temporal Shh gradient in specifying vertebrate digit identities. *Cell.* **118**, 517–528.
31. Kavanagh, K. D., Evans, A. R., and Jernvall, J. (2007) Predicting evolutionary patterns of mammalian teeth from development. *Nature.* **449**, 427–432.
32. Munne, P., Tümmers, M., Järvinen, E., Thesleff, I., and Jernvall, J. (2009) Tinkering with the inductive mesenchyme: *Sostdc1* uncovers the role for dental mesenchyme in limiting tooth induction. *Development.* **136**, 393–402.

Chapter 17

A Method to Isolate, Purify, and Characterize Human Periodontal Ligament Stem Cells

Krzysztof Mrozik, Stan Gronthos, Songtao Shi, and P. Mark Bartold

Abstract

Human periodontal ligament stem cells (PDLSCs) are a unique population of mesenchymal stem cells (MSCs) which demonstrate the capacity to generate cementum- and periodontal ligament-like structures *in vivo*. As such, PDLSCs represent a promising cell-based therapy in reconstructive dentistry for the treatment of periodontal disease. The present chapter describes two methods for isolating PDLSCs from human PDL tissue including traditional plastic adherence and immunomagnetic selection based on the expression of MSC-associated surface markers STRO-1 antigen, CD146 (MUC-18), CD29 (integrin β -1), CD44, and CD106 (VCAM-1). Although no single antibody demonstrates specificity for MSCs, isolation based on the expression of individual markers results in homogeneous preparations of PDLSCs. Methods to further characterize the immunophenotype and multipotent capacity of PDLSCs to differentiate into adipocytes, osteoblast- and cementoblast-like cells *in vitro*, and cementum- and periodontal ligament-like tissues *in vivo* are also described.

Key words: Periodontal ligament stem cells, mesenchymal stem cells, adherence isolation, immunomagnetic isolation, differentiation potential.

1. Introduction

The presence of multiple cell types (fibroblasts, cementoblasts, and osteoblasts) within the postnatal periodontal ligament suggests that these cells may share common ancestors. The possibility that progenitor cells might exist in the postnatal periodontal ligament has been recognized for some time but until recently had never been formally proven (1). These cells are believed to

provide a renewable cell source for normal tissue homeostasis and periodontal wound healing (2, 3).

Recently, multipotent stem cell populations, termed periodontal ligament stem cells (PDLSCs), have been isolated from the periodontal ligament of extracted human third molar teeth (4). These PDLSCs give rise to adherent clonogenic clusters that resemble fibroblasts and are capable of developing into adipocytes, osteoblast- and cementoblast-like cells in vitro, and demonstrate the capacity to produce cementum- and periodontal ligament-like tissues in vivo (4–6). PDLSCs express an array of cementoblast and osteoblast markers as well as several bone marrow-derived mesenchymal stem/stromal cell (MSC)-associated markers (7, 8). The similarity between PDLSCs and bone marrow MSCs suggests that PDLSCs represent another MSC-like population. Further work is now focusing on identifying markers uniquely expressed by PDLSCs to discriminate these cells from other types of MSC-like cells identified in dental tissues (9).

The first reported isolation and identification of MSCs in human periodontal ligament was in 2004 (4). Since then, there has been considerable activity trying to understand the function of these cell populations and their interactions with each other with a view to laying the fundamental groundwork for clinical applications in regenerative periodontics. A number of studies have now been carried out to confirm the presence of MSC-like cells in the periodontal ligament. These have not been limited to human but also include mouse, rat, and sheep (5, 8, 10–14). All of these studies have confirmed the multipotent nature of PDLSCs, and while the initial studies indicated this to include an ability to differentiate into osteoblast, cementoblast, or adipogenic phenotypes, at least one recent study has indicated an ability of these cells to also differentiate into neuronal precursors (14). Importantly, cryopreservation does not seem to alter the functional properties of PDLSCs (15). This will have significant relevance should “banking” of these cells become a clinical necessity.

Identification of stem cells in postnatal dental tissues has presented exciting possibilities for the application of tissue engineering as well as gene- and cell-based therapies in reconstructive dentistry. The use of stem cells with these technologies may constitute novel strategies for regenerative periodontal therapy. Periodontitis is a disease of the periodontium characterized by irreversible loss of connective tissue attachment and supporting alveolar bone (16). These changes often lead to an aesthetically and functionally compromised dentition. For many decades, periodontists have been interested in regenerating tissues destroyed by periodontitis. Periodontal regeneration can be defined as the complete restoration of the lost tissues to their original architecture and function by recapitulating the crucial wound

healing events associated with their development (17, 18). The isolation of adult stem cells from human periodontal ligament has presented new opportunities for tissue engineering (5, 10). Clearly, in order for such therapies to be successful, suitable means of procuring these cells and expanding them in vitro are essential. This chapter describes methods for the isolation, ex vivo expansion, and characterization of PDLSCs from human periodontal ligament.

2. Materials

2.1. Processing of Periodontal Ligament

1. Wash buffer [Hanks' balanced salt solution (HBSS; JRH Biosciences, Lenexa, KS, USA) supplemented with 5% (v/v) fetal bovine serum (FBS; JRH Biosciences) and 50 U/mL penicillin and 50 µg/mL streptomycin].
2. Type I collagenase [6 mg/mL stock solution in PBS (phosphate buffered saline)].
3. Dispase II (8 mg/mL stock solution in PBS).
4. White cell fluid; 2% acetic acid in distilled H₂O.
5. 70-µm Cell strainer.
6. 10-cm Tissue culture dish.
7. 14-mL Polypropylene round-bottom tube.
8. Forceps.
9. Scalpel handle size 3.
10. Surgical blade size 11.

2.2. Dynal Immunomagnetic Cell Isolation and Fluorescence-Activated Cell Sorting

1. Blocking buffer [HBSS supplemented with 5% FBS, 5% normal human serum (NHS; AB⁺, *see Note 1*), 1% bovine serum albumin (BSA), 50 U/mL penicillin, and 50 µg/mL streptomycin].
2. Wash buffer (*see above*).
3. Murine monoclonal primary antibodies (anti-human): (a) STRO-1 (IgM, anti-human stromal cell; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), (b) anti-CD29 (IgG₁; BD Biosciences, San Jose, CA, USA), (c) H9H11 (IgG₁, anti-CD44; A/Prof. Andrew Zannettino, Division of Hematology, IMVS, Adelaide, SA, Australia) (19), (d) 6G10 (IgG₁, anti-CD106, VCAM-1; American Type Culture Collection Manassas, VA. ATCC No. HB 10519), (e) CC9 (IgG_{2a}, anti-human CD146, MUC-18; A/Prof. Stan Gronthos, Division of Hematology, IMVS, Adelaide, SA, Australia)

- (20, 21), (f) CD166 (ALCAM; BD Biosciences), (g) anti-CD105 (IgG₁; BD Biosciences), (h) anti-CD166 (IgG₁; BD Biosciences), (i) anti-CD14 fluorescein isothiocyanate (FITC) conjugated (IgG_{2a}; Beckman Coulter, Fullerton, CA, USA), (j) anti-CD31 FITC conjugated (IgG₁; Beckman Coulter), (k) anti-CD45 FITC conjugated (IgG₁; Beckman Coulter).
4. Isotype-matched control: (a) 1B5 (IgG₁), (b) 1A6.11 (IgG₂), (c) 1A6.12 (IgM) (Prof L.K. Ashman; The University of Newcastle, Newcastle, NSW, Australia). Isotype-matched controls are also commercially available.
 5. Dynabead[®]-conjugated rat anti-mouse IgM and goat anti-mouse IgG secondary antibodies (DynaL Biotech ASA, Oslo, Norway).
 6. Goat anti-mouse IgM-FITC-conjugated antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) and goat anti-mouse IgG-FITC-conjugated antibody (Southern Biotechnology Associates, Inc.).
 7. Dynal MPC[®]-2 Magnetic Particle Concentrator (DynaL Biotech ASA).
 8. FACS fix: 1% (v/v) Formalin, 0.1 M D-glucose, 0.02% sodium azide in PBS.
 9. 14-mL Polypropylene round-bottom tube.
 10. 5-mL Polypropylene round-bottom tube.

2.3. Cell Culture of Human PDLSC

1. α -MEM growth medium [α -MEM; JRH Biosciences, Lenexa, KS, USA) supplemented with 20% (v/v) FBS, 2 mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 μ g/mL streptomycin].
2. Hanks' balanced salt solution (HBSS; JRH Biosciences, Lenexa, KS, USA).
3. Osteogenic inductive medium [α -MEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 10^{-7} M dexamethasone, 1.8 mM inorganic phosphate (KH₂PO₄), 50 U/mL penicillin, and 50 μ g/mL streptomycin].
4. Adipogenic inductive medium [α -MEM supplemented with 10% (v/v) FBS, 0.5 mM isobutylmethylxanthine, 60 μ M indomethacin, 0.5 μ M hydrocortisone, 15 mM Hepes buffer, 2 mM L-glutamine, 100 μ M L-ascorbate-2-phosphate, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 μ g/mL streptomycin].
5. Phosphate buffered saline solution, pH 7.4.
6. 0.5% Trypsin/0.2% EDTA solution (10 $\times$ stock solution).

7. 0.4% Trypan blue/PBS.
8. 0.1% (w/v) Toluidine blue.
9. T-25 and T-75 culture flasks.
10. Six-well culture plates.
11. 14-mL Polypropylene round-bottom tubes.
12. 1.8-mL Cryotubes.
13. Freeze mix [20% dimethyl sulfoxide (DMSO) in FBS].
14. Cryo 1°C freezing container “Mr. Frosty” (Nalge Nunc International).
15. Alizarin red stain [1% alizarin red (Sigma-Aldrich, St. Louis, MO), 2% ethanol in distilled water].
16. Oil red O stain [0.5 g oil red O (ICN Biomedicals, Inc., Aurora, Ohio) stain dissolved in 100 mL isopropanol and mixed in 3:2 ratio with distilled water].
17. 1% (w/v) Paraformaldehyde (PFA) in PBS.
18. 10% Neutral buffered formalin.

**2.4. Attachment
of PDLSCs to HA/TCP
Particles and
Subcutaneous
Implantation**

1. Hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic particles (Zimmer Corporation, Warsaw, IN).
2. Fibrinogen (Sigma-Aldrich).
3. Thrombin (Sigma-Aldrich).
4. Surgical scissors.
5. AUTOCLIP® Wound Clips 9 mm (BD Biosciences).
6. AUTOCLIP® Applier 9 mm (BD Biosciences).

**2.5. Recovery
of Transplant,
Processing, and
Immunohisto-
chemistry**

1. 4% Paraformaldehyde (4 g paraformaldehyde in PBS).
2. 10% EDTA (ethylenediaminetetraacetic acid in deionized water).
3. Ethanol.
4. Xylene.
5. Paraffin wax.
6. Forceps.
7. Scalpel handle size 3.
8. Surgical blade size 11.
9. Mayer’s hematoxylin (Lillie’s modification).
10. Acid alcohol: 0.3% Concentrated hydrochloric acid (HCl), 70% ethanol in distilled water.
11. Bicarbonate solution (1% in distilled water).
12. Eosin: 0.114% Eosin Y, 0.0114% aqueous phloxine, 0.46% glacial acetic acid (v/v), 84.1% ethanol in distilled water.

13. Gurr's DePeX mounting medium.
14. 30% Hydrogen peroxide.
15. Sodium azide.
16. Goat serum.
17. Rabbit polyclonal primary antibodies: (a) Bone sialoprotein (BSP) LF-120 (Dr. Larry Fisher, Craniofacial and Skeletal Diseases Branch, NIDCR/NIH, Bethesda, MD, USA) (22), (b) osteocalcin (OSC) LF-32 (Dr. Larry Fisher, Craniofacial and Skeletal Diseases Branch, NIDCR/NIH, Bethesda, MD, USA) (23).
18. Rabbit Ig control (Caltag Laboratories, Burlingame, CA; Code No. 10500).
19. Secondary antibody: Biotinylated goat anti-rabbit IgG (H+L) antibody (Vector Laboratories, Inc., Burlingame, CA).
20. Vectastain® ABC Kit (avidin–horseradish peroxidase kit; Vector Laboratories, Inc.).
21. AEC kit (horseradish peroxidase substrate; Vector Laboratories, Inc.).

3. Methods

3.1. Processing of Human Periodontal Ligament (PDL)

3.1.1. Collection of Periodontal Ligament Cells

Following informed consent, teeth with healthy periodontal ligament can be obtained as a result of tooth extraction for orthodontic purposes or removal of third molars. PDL tissue can be pooled from multiple teeth and from different donors, provided they are all processed within 2 h of extraction.

1. Gently separate the periodontal ligament (PDL) from the middle third of the tooth root surface using forceps and a size 11 surgical blade in a 10-cm tissue culture dish containing 10 mL wash buffer.
2. Transfer wash buffer containing PDL tissue into a 14-mL round-bottom tube and centrifuge at $400\times g$ for 10 min at 4°C.
3. Resuspend and digest PDL in a solution of 1 mL type I collagenase (3 mg/mL final) and 1 mL dispase II (4 mg/mL final) for 1 h at 37°C (*see Note 2*).

4. Add an excess volume of wash buffer to the digested tissue to neutralize enzyme activity and then strain through a 70- μ m cell strainer to remove undigested tissue from the liberated periodontal ligament cells.
5. Pellet PDL cells by centrifugation at $400\times g$ for 10 min at 4°C, resuspend in 2 mL wash buffer, and keep on ice.
6. Remove a 10 μ L aliquot and dilute 1:10 into white cell fluid (WCF) and enumerate nucleated cells using a hemocytometer (*see Note 3*). Typically, the PDL cell count ranges from 0.5 to 2.5×10^4 per one to three teeth processed.

3.2. Isolation of Periodontal Ligament Stem Cells

Single-cell suspensions generated from digested human PDL tissue have the capacity to form adherent clonogenic cell clusters with a fibroblast-like morphology in an *in vitro* culture setting. Each colony originates from a single progenitor cell (colony-forming unit–fibroblast, CFU-F), similarly to colonies formed by dental pulp stem cells (DPSCs) and bone marrow-derived MSCs (BM MSCs) (4, 5, 7, 20, 24). The colony-forming cell population residing in PDL tissue has been termed periodontal ligament stem cells (PDLSCs) (4). However, traditional methods of isolating bone marrow-derived CFU-F based on plastic adherence result in a heterogeneous population whereby some individually expanded colonies display a multipotent capacity for lineage differentiation, whereas others demonstrate a restricted differentiation potential (24–27).

Homogeneous populations of PDLSCs and other MSCs can be immunomagnetically isolated according to the particular markers expressed on the cell surface. Although no single antigenic marker demonstrates specificity for MSCs, *ex vivo*-expanded PDLSCs express an unidentified early mesenchymal stem cell-associated surface antigen reactive to the antibody STRO-1. Similar to DPSCs and BM MSCs, STRO-1 antibody-based isolation of PDLSCs released from freshly digested PDL tissue demonstrates that the majority of colony-forming units are contained within the STRO-1⁺ fraction. Thus, the reactive antigen to the STRO-1 antibody is considered an important MSC-associated marker expressed by PDLSCs (4). Further discrimination of the STRO-1⁺ DPSC and BM MSC fractions has been achieved by purification based on their expression of endothelial cell markers CD146 (MUC18) and CD106 (VCAM-1), demonstrating their localization within the perivascular niche (7, 20, 26, 27). Although PDLSCs also express CD146 and CD106 implying a perivascular origin (4, 5, 9), the relatively small number of cells released from PDL tissue compared to dental pulp and bone marrow is a significant limitation in applying a dual-antibody isolation approach. However, positive isolation of PDLSCs can also be achieved using antibodies to various other MSC-associated

markers including CD106 (5), CD29 (integrin β -1) (7); CD44 (7, 9); and CD146 (4, 9) as stand-alone reagents. Similar to STRO-1, these antibodies positively select the majority of colony-forming units within PDL tissue with varying efficiencies.

3.2.1. Adherence Isolation of Periodontal Ligament Stem Cells and Ex Vivo Culture

1. Single-cell suspensions generated from digested PDL are initially plated in T-25 culture flasks or six-well plates in α -MEM growth medium. Cultures are incubated at 37°C in 5% CO₂ and >90% humidity (*see Note 4*).
2. Adherent primary PDLSC colonies (CFU-F) are “passaged” when 70–80% confluency is achieved after approximately 2 weeks. At this point in time, PDLSC cultures are washed once with HBSS and liberated by enzymatic digestion by the addition of 1 mL of 0.05% trypsin/0.02% EDTA solution per T-25 culture flask or six-well plate for 5–10 min at 37°C. The single-cell suspension is then washed twice in wash buffer using a 14-mL tube (*see Note 5*).
3. Cell viability is assessed by removing a 10 μ L aliquot of the single-cell suspension and diluting 1:10 in 0.4% trypan blue/PBS. The number of viable cells can be enumerated using a hemocytometer (non-viable cells take up the blue dye).
4. To expand cultures, PDLSCs are re-seeded (passaged) into T-75 culture flasks at $5\text{--}8 \times 10^3$ cells/cm² in α -MEM growth medium. Cultures are refed twice weekly by aspirating the growth medium and replacing with an equal volume of fresh growth medium warmed to 37°C (*see Note 5*).

3.2.2. Immunomagnetic Isolation of Periodontal Ligament Stem Cells and Ex Vivo Culture

1. Resuspend single-cell suspensions generated from digested PDL in 10 mL blocking buffer using a 14-mL round-bottom tube and incubate on ice for 30 min to reduce the possibility of Fc receptor-mediated binding of antibodies.
2. Pellet cells by centrifugation at $400 \times g$ for 10 min at 4°C and resuspend all cells (usually $0.5\text{--}2.5 \times 10^4$ cells) in 1 mL of desired primary antibody [STRO-1 (IgM) and anti-CD146 (IgG_{2a}) monoclonal supernatants (1/2 dilution) or purified IgG₁ monoclonal antibodies anti-CD29, anti-CD44, and anti-CD106 (10 μ g/mL)] using a 5-mL tube and incubate on ice for 1 h with occasional, gentle mixing.
3. Wash cells twice in wash buffer by centrifugation at $400 \times g$ for 10 min at 4°C and resuspend in 1 mL of appropriate Dynabead[®]-conjugated secondary antibody (*see Note 6*), mix, and incubate on ice for 10 min. Add 2.5 mL wash buffer and incubate for 2 h at 4°C on rotator. Check for beads bound to cells microscopically.

4. Mix the cell suspension and place the tube in a Dynal MPC®-2 Magnetic Particle Concentrator for 2 min. Aspirate off cells that are not bound to the magnet (negative fraction), add 3 mL cold wash buffer to bead-bound cells, mix the cell suspension, again place the tube in the magnetic particle concentrator, and aspirate unbound cells. Repeat this process until there are no unbound cells remaining in the suspension. This can be checked microscopically.
5. At this point, purified PDLSCs can be culture-expanded similar to PDL cells following digestion as described for adherence isolation of PDLSCs (*see* **Section 3.2.1**) or used in colony-forming unit–fibroblast (CFU-F) efficiency assays as described in **Section 3.3**.

3.2.3. Cryopreservation of Ex Vivo-Expanded PDLSCs

1. Single-cell suspensions of culture-expanded PDLSCs are prepared by 0.05% trypsin/0.02% EDTA digestion and cells enumerated and viability assessed using 0.4% trypan blue/PBS as described above.
2. Cells are resuspended at a concentration of 4×10^6 cells/mL of FBS and kept on ice. An equal volume of ice-cold freeze mix is then added drop-wise while gently agitating the cells to give a final concentration of 2×10^6 cells/mL in 10% DMSO/FBS. Aliquots of 1 mL are distributed into 1.8-mL cryovials pre-cooled on ice and then frozen at a rate of approximately $-1^\circ\text{C}/\text{min}$ using a Cryo 1°C freezing container “Mr. Frosty” pre-cooled to 4°C . Place the container holding the cryovials at -80°C overnight before transferring the cryovials into liquid nitrogen for long-term storage.
3. Recovery of the cryopreserved stock is achieved by rapidly thawing the cells in a 37°C water bath (*see* **Note 7**). Resuspend the cells in 20 mL cold wash buffer and spin at $280 \times g$ for 10 min.
4. Assess viability of cells using 0.4% trypan blue/PBS as described above. Typically this procedure results in cell viabilities between 80 and 90%.

3.3. Assessment of Colony-Forming Unit–Fibroblast (CFU-F) Efficiency Assay

1. Single-cell suspensions harvested from freshly digested PDL or immunomagnetically isolated PDLSCs are seeded in triplicate into six-well culture plates at $0.25\text{--}1.0 \times 10^4$ cells per well in α -MEM growth medium. Cultures are incubated at 37°C in 5% CO_2 and $>90\%$ humidity for 12 days (*see* **Note 8**).
2. Cultures are washed twice with PBS and then fixed for 20 min in 1% (w/v) PFA in PBS.

3. Cultures are stain fixed with 0.1% (w/v) toluidine blue (in 1% PFA) for 1 h, then rinsed with tap water, and allowed to dry.
4. Aggregates of greater than 50 cells are scored as CFU-F using either an inverted or a dissecting light microscope.

3.4. Flow-Cytometric Analysis of PDLSCs

To characterize the immunophenotype of ex vivo-expanded PDLSCs, flow-cytometric analysis can be used to measure the expression of mesenchymal and non-mesenchymal stem cell-associated surface markers at early passages. The relatively low number of cells initially harvested from the digestion of PDL tissue ($<2.5 \times 10^4$) is usually inadequate to perform flow-cytometric analysis without ex vivo expansion.

1. Adherent ex vivo-expanded PDLSCs are washed once with HBSS and liberated by enzymatic digestion by the addition of 3 mL of 0.05% trypsin/0.02% EDTA solution per T-75 culture flask for 5–10 min at 37°C. The single-cell suspension is then washed twice in wash buffer.
2. Cell count and assessment of viability is performed as described above.
3. PDLSCs are resuspended for immunolabeling in 0.5 mL blocking buffer and incubated on ice for approximately 30 min to reduce the possibility of Fc receptor-mediated binding of antibodies.
4. Individual 5-mL polypropylene round-bottom tubes containing 1×10^5 PDLSCs are incubated with appropriate primary murine monoclonal antibodies: STRO-1, anti-CD29, anti-CD44, anti-CD105, anti-CD106, anti-CD146, anti-CD166 (mesenchymal stem cell-associated markers), anti-CD14, anti-CD31, anti-CD45 (hematopoietic, non-mesenchymal stem cell-associated markers), or isotype-matched control at a concentration of 20 µg/mL for 1 h on ice. Wash the cells twice in 1 mL wash buffer.
5. Cells are incubated with secondary detection reagents such as goat anti-mouse IgG- or goat anti-mouse IgM-FITC (fluorescein isothiocyanate)-conjugated antibody (1:50; Southern Biotechnology, Birmingham, AL) for 45 min on ice (*see Note 9*). The cells are washed twice in 1 mL.
6. FACS fix solution (0.5 mL) is added to each tube to fix the cells.
7. The cells are analyzed on any fluorescence-activated cell sorter fitted with a 250-MW argon laser-emitting light at a wavelength of 488 nm able to detect FITC (or other fluorophores).

3.5. Differentiation Potential of PDLSCs In Vitro

The capacity of mesenchymal stem cells to generate stromal tissues including those similar to which they were derived from is recognized as a hallmark feature of these cells. The ability of PDLSCs to differentiate into various stromal cell lineages in vitro can be investigated by culturing under inductive conditions.

3.5.1. In Vitro Formation of Bone Mineral

1. Seed 1×10^5 in vitro-expanded PDLSCs per T-25 culture flask in 5 mL α -MEM growth medium and incubate at 37°C in 5% CO₂ and >90% humidity.
2. After 24 h, aspirate the α -MEM growth medium and add an equivalent volume of osteogenic inductive medium. Replace the osteogenic inductive medium twice a week.
3. After 4 weeks, aspirate the medium and gently rinse the osteogenic-induced culture with PBS five times, fix with 10% neutral buffered formalin for 1 h at room temperature (RT), and then rinse three times with distilled H₂O.
4. Stain the osteogenic-induced culture with 1% alizarin red, 2% ethanol in distilled H₂O for 1 h at RT. Mineralized deposits of calcium will appear red (*see Note 10*).

3.5.2. In Vitro Differentiation into Adipocytes

1. Seed 5×10^4 in vitro-expanded PDLSCs per well using a 24-well plate in 500 μ L α -MEM growth medium and incubate at 37°C in 5% CO₂ and >90% humidity.
2. After 24 h, aspirate the α -MEM growth medium and add an equivalent volume of adipogenic inductive medium. Replace the adipogenic inductive medium twice a week.
3. After 4 weeks, aspirate the medium and gently rinse the adipogenic-induced culture once with PBS and fix with 10% neutral buffered formalin for 10 min at RT.
4. Aspirate the formalin and stain the adipogenic-induced culture with oil red O stain for at least 2 h at RT. Lipid-laden vacuoles within adipocytes will appear red (*see Note 11*).

3.6. Differentiation Potential of PDLSCs In Vivo

In order to demonstrate that ex vivo-expanded PDLSCs can differentiate into functional cementoblast- or osteoblast-like cells, cells attached to osteogenic-conductive hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic carrier particles can be subcutaneously transplanted into immunocompromised mice (*see Note 12*).

3.6.1. Attachment of PDLSCs to HA/TCP Particles

1. Prepare single-cell suspensions of ex vivo-expanded PDLSCs following 0.5% trypsin/EDTA digestion and assess cell viability using 0.4% trypan blue/PBS as described above.
2. Resuspend 5×10^6 ex vivo-expanded PDLSCs in 1 mL α -MEM growth medium and transfer to a 1.8-mL cryovial

containing 40 mg HA/TCP ceramic carrier particles (Zimmer, Warsaw, IN) (*see Note 13*).

3. Gently mix the cell suspension and HA/TCP particles using a rotator while incubating at 37°C for 1 h to enhance cell attachment to the particles.
4. Gently pellet the mix at 300×*g* for 2 min and discard the supernatant.
5. Approximately 10 min prior to implantation, add 20 µL mouse fibrinogen (30 mg/mL in PBS) and 20 µL mouse thrombin (100 U/mL in 2% CaCl₂) to the cells attached to HA/TCP ceramic carrier particles and gently mix in using a pipette tip to form a plug.

3.6.2. Subcutaneous Implantation Procedure

1. In 6–10-week-old immunocompromised NOD/SCID mice, perform a 1-cm mid-longitudinal skin incision on the dorsal surface and create a subcutaneous pocket by blunt dissection.
2. Place the polymerized transplant into the subcutaneous pocket and close the incision with AUTOCLIP® 9-mm Wound Clips using AUTOCLIP® 9-mm Applier (*see Note 14*).

3.6.3. Recovery of Transplants, Processing, and Hematoxylin and Eosin Staining

1. Recover the transplants 8 weeks after transplantation, cut into two pieces using a surgical blade, and fix in 4% paraformaldehyde for 2 days.
2. Decalcify transplant for 10 days in 12 mL of 10% EDTA solution using a 14-mL round-bottom tube while rotating (*see Note 15*).
3. Process transplants by dehydration through an increasing gradient of ethanol concentrations (50, 70, 90% and several changes in 100%) and then three changes in xylene. Wash transplants twice in molten paraffin wax before embedding in molten paraffin wax. Allow to cool to form a block and prepare 5-µm sections.
4. Deparaffinize sections in xylene (2 × 5 min) and then rehydrate through a decreasing gradient of ethanol concentrations (5 min each; 2 × 100%, 1 × 90%, 1 × 70%, 1 × 50%, and 2 × distilled water).
5. Stain with Mayer's hematoxylin (Lillie's modification) for 5 min, wash off hematoxylin in running tap water, and then rinse in distilled water for 10 s. Immerse in bicarbonate solution for 10 s, wash in running tap water, decolorize in 0.3% acid alcohol for 5 s, and again wash in running tap water. Blue the sections in lithium carbonate and wash in running tap water. Counterstain sections in eosin for 2 min and dehydrate in three changes of 100% ethanol (30 s each).

Immerse in two changes of xylene for 30 s each and mount in Gurr's DePeX mounting medium.

3.6.4.
Immunohistochemistry

1. Sections are deparaffinized in xylene (2×5 min) and then rehydrated through a decreasing gradient of ethanol concentrations (5 min each; $2 \times 100\%$, $1 \times 90\%$, $1 \times 70\%$, $1 \times 50\%$, and $2 \times$ distilled water).
2. Endogenous peroxidase activity is blocked using 0.5% hydrogen peroxide diluted in 0.1% sodium azide and PBS for 20 min.
3. Sections are rinsed three times in PBS for 5 min and blocked in 5% goat serum for 1 h at RT.
4. Primary rabbit polyclonal antibodies are diluted in 5% goat serum [1:500; bone sialoprotein (BSP, LF-120), osteocalcin (OSC, LF-32) and equivalent concentration of rabbit Ig control], added to each slide, and incubated for 2 h at RT.
5. Sections are washed three times in PBS (5 min per wash), then incubated with the secondary antibody goat anti-rabbit Ig-biotinylated antibody (1/100 dilution) for 60 min, before washing three times in PBS.
6. Avidin-horseradish peroxidase conjugate (Vectastain® ABC Kit) is then prepared as recommended by the manufacturer and added to the sections for 30 min at room temperature.
7. After three washes in PBS, horseradish peroxidase substrate [AEC (3-amino 9-ethylcarbazole) kit] is added to the sections according to the manufacturer's protocol and incubated until color development has occurred (*see Note 16*).
8. Sections are washed three times with distilled water, counterstained with Mayer's hematoxylin (Lillie's Modification) for 2 min, dehydrated in three changes of 100% ethanol (30 s each), immersed in two changes of xylene for 30 s each, and mounted in Gurr's DePeX mounting medium.

4. Notes

1. Prior to use, normal human serum should be heat inactivated at 56°C for 30 min in a shaking water bath, then centrifuged at $1,000 \times g$ for 10 min, and supernatant collected.
2. The stated volume of type I collagenase and dispase II is adequate for processing up to four teeth.
3. Wipe any excess cell suspension on pipette tip with tissue before mixing with white cell fluid or 0.4% trypan

blue/PBS to ensure that the cell count number is not overestimated.

4. Store growth medium at 4°C. If greater than 1 week old at 4°C, add fresh 2 mM L-glutamine prior to use.
5. If adherent cell cultures are overconfluent, wash cells once with HBSS and incubate with equal volume of type I collagenase (3 mg/mL final) and dispase II (4 mg/mL final) (1 mL total per 25 cm² surface area) for 1 h at 37°C. Wash the liberated single-cell suspension twice in wash buffer. If cells appear to be clumped, pass them through a 70-μm cell strainer prior to re-seeding.
6. Prior to incubation, the Dynabead-conjugated secondary antibody should be washed to remove immunoglobulins not attached to beads. Add the required volume of Dynabeads (calculate volume based on four beads per cell) to 3 mL wash buffer, mix the suspension, and place the tube in a DYNAL MPC®-2 Magnetic Particle Concentrator for 2 min. Aspirate off wash buffer containing unbound immunoglobulins, resuspend beads in fresh wash buffer, and leave on ice until required.
7. Do not heat thawing cells to 37°C. Remove the cryotube from the water bath as soon as the sample is thawed.
8. Check for overgrowth of CFU-F at day 10 to prevent colonies growing into each other.
9. Phycoerythrin (PE)-conjugated secondary antibodies can also be used.
10. Rinse flasks with distilled H₂O until excess alizarin red stain is removed.
11. Rinse flasks with distilled H₂O until excess oil red O stain is removed and store in distilled H₂O at 4°C (do not allow to air-dry).
12. This procedure requires animal ethics approval from the appropriate body and should be performed in accordance with specifications of an approved small-animal protocol.
13. Prior to cell attachment, pre-wash HA/TCP particles in 1.5 mL wash buffer on rotator at 37°C for 1 h. Remove wash buffer before addition of cell suspension.
14. Up to four transplants can be performed per animal (one transplant per subcutaneous pocket created).
15. Change 10% EDTA solution daily and confirm completion of decalcification by X-ray analysis.
16. Steps 5 and 6 can be substituted using a broad-spectrum immunoperoxidase AEC (Rabbit) staining kit (Invitrogen, Carlsbad, CA, USA).

References

- Melcher, A. H. (1985) Cells of periodontium: their role in the healing of wounds. *Ann. R. Coll. Surg. Engl.* **67**, 130–131.
- Pitaru, S., McCulloch, C. A., and Narayanan, S. A. (1994) Cellular origins and differentiation control mechanisms during periodontal development and wound healing. *J. Periodont. Res.* **29**, 81–94.
- Gould, T. R., Melcher, A. H., and Brunette, D. M. (1980) Migration and division of progenitor cell populations in periodontal ligament after wounding. *J. Periodont. Res.* **15**, 20–42.
- Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahimi, J., Young, M., Robey, P. G., Wang, C. Y., and Shi, S. (2004) Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet.* **364**, 149–155.
- Gronthos, S., Mrozik, K., Shi, S., and Bartold, P. M. (2006) Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. *Calcif. Tissue Int.* **79**, 310–317.
- Shi, S., Bartold, P. M., Miura, M., Seo, B. M., Robey, P. G., and Gronthos, S. (2005) The efficacy of mesenchymal stem cells to regenerate and repair dental structures. *Orthod. Craniofac. Res.* **8**, 191–199.
- Gronthos, S., Mankani, M., Brahimi, J., Robey, P. G., and Shi, S. (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc. Natl. Acad. Sci. USA.* **97**, 13625–13630.
- Trubiani, O., Di Primio, R., Traini, T., Pizzicannella, J., Scarano, A., Piattelli, A., and Caputi, S. (2005) Morphological and cytofluorimetric analysis of adult mesenchymal stem cells expanded ex vivo from periodontal ligament. *Int. J. Immunopathol. Pharmacol.* **18**, 213–221.
- Chen, S. C., Marino, V., Gronthos, S., and Bartold, P. M. (2006) Location of putative stem cells in human periodontal ligament. *J. Periodont. Res.* **41**, 547–553.
- Nagatomo, K., Komaki, M., Sekiya, I., Sakaguchi, Y., Noguchi, K., Oda, S., Muneta, T., and Ishikawa, I. (2006) Stem cell properties of human periodontal ligament cells. *J. Periodont. Res.* **41**, 303–310.
- Jo, Y. Y., Lee, H. J., Kook, S. Y., Choung, H. W., Park, J. Y., Chung, J. H., Choung, Y. H., Kim, E. S., Yang, H. C., and Choung, P. H. (2007) Isolation and characterization of postnatal stem cells from human dental tissues. *Tissue Eng.* **13**, 767–773.
- Ivanovski, S., Haase, H. R., and Bartold, P. M. (2001) Isolation and characterization of fibroblasts derived from regenerating human periodontal defects. *Arch. Oral Biol.* **46**, 679–688.
- Luan, X., Ito, Y., Dangaria, S., and Diekwisch, T. G. (2006) Dental follicle progenitor cell heterogeneity in the developing mouse periodontium. *Stem Cells Dev.* **15**, 595–608.
- Techawattanawisal, W., Nakahama, K., Komaki, M., Abe, M., Takagi, Y., and Morita, I. (2007) Isolation of multipotent stem cells from adult rat periodontal ligament by neurosphere-forming culture system. *Biochem. Biophys. Res. Commun.* **357**, 917–923.
- Seo, B. M., Miura, M., Sonoyama, W., Coppe, C., Stanyon, R., and Shi, S. (2005) Recovery of stem cells from cryopreserved periodontal ligament. *J. Dent. Res.* **84**, 907–912.
- Pihlstrom, B. L., Michalowicz, B. S., and Johnson, N. W. (2005) Periodontal diseases. *Lancet.* **366**, 1809–1820.
- Polimeni, G., Xiropaidis, A. V., and Wikesjo, U. M. (2006) Biology and principles of periodontal wound healing/regeneration. *Periodontol 2000.* **41**, 30–47.
- Lin, N. H., Gronthos, S., and Bartold, P. M. (2008) Stem cells and periodontal regeneration. *Aust. Dent. J.* **53**, 108–121.
- Zannettino, A. C., Paton, S., Arthur, A., Khor, F., Itescu, S., Gimble, J. M., and Gronthos, S. (2008) Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J. Cell. Physiol.* **214**, 413–421.
- Shi, S., and Gronthos, S. (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J. Bone Miner. Res.* **18**, 696–704.
- Filshie, R. J., Zannettino, A. C., Makrynika, V., Gronthos, S., Henniker, A. J., Bendall, L. J., Gottlieb, D. J., Simmons, P. J., and Bradstock, K. F. (1998) MUC18, a member of the immunoglobulin superfamily, is expressed on bone marrow fibroblasts and a subset of hematological malignancies. *Leukemia.* **12**, 414–421.
- Fedarko, N. S., Fohr, B., Robey, P. G., Young, M. F., and Fisher, L. W. (2000) Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. *J. Biol. Chem.* **275**, 16666–16672.
- Ingram, R. T., Clarke, B. L., Fisher, L. W., and Fitzpatrick, L. A. (1993) Distribution

- of noncollagenous proteins in the matrix of adult human bone: evidence of anatomic and functional heterogeneity. *J. Bone Miner. Res.* **8**, 1019–1029.
24. Kuznetsov, S. A., Krebsbach, P. H., Sato-mura, K., Kerr, J., Riminucci, M., Benayahu, D., and Robey, P. G. (1997) Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo. *J. Bone Miner. Res.* **12**, 1335–1347.
25. Muraglia, A., Cancedda, R., and Quarto, R. (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J. Cell Sci.* **113**, 1161–1166.
26. Gronthos, S., Brahimi, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., DenBesten, P., Robey, P. G., and Shi, S. (2002) Stem cell properties of human dental pulp stem cells. *J. Dent. Res.* **81**, 531–535.
27. Gronthos, S., Zannettino, A. C., Hay, S. J., Shi, S., Graves, S. E., Kortesidis, A., and Simmons, P. J. (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J. Cell Sci.* **116**, 1827–1835.

Chapter 18

Preclinical Methods for the Evaluation of Periodontal Regeneration In Vivo

Yang-Jo Seol, Gaia Pellegrini, Lea M. Franco, Po-Chun Chang, Chan Ho Park, and William V. Giannobile

Abstract

For the determination of key factors or devices that promote periodontal regeneration, preclinical investigations using in vivo animal models are critical for evaluating the biological responses before human clinical trial testing. In this chapter, we provide an overview on the commonly used preclinical animals for the study of reconstructive procedures to promote bone and soft tissue repair of tooth-supporting periodontal defects. Steps are provided on the animal management for evaluation of outcome measures using descriptive histology, histomorphometry, three-dimensional imaging, and safety assessments. The use of these key measures of periodontal regeneration should aid investigators in the selection of appropriate surrogate endpoints to be utilized in the clinical arena, which are not practical or ethical in humans. These methods will prepare investigators and assist them in identifying endpoints that can then be adapted to human clinical trial planning.

Key words: Periodontal regeneration, tissue engineering, bioactive molecules, animal surgery, rat, canine.

1. Introduction

There are many critical steps involved in the appropriate design and implementation of investigations for determination of the safety and efficacy of regenerative devices and biologics for periodontal repair. The first phase is molecule discovery, performed via in vitro experiments based on biological plausibility. A variety of bioactive molecules and osteoconductive scaffolding matrices have been demonstrated to be effective to enhance cell activity

such as proliferation, differentiation, and matrix biosynthesis (1–5). After confirming safety and efficacy within animals, the planning and design for human clinical trials can begin. Thus, animal experimentation is a necessary bridge from the laboratory to the clinic. In the preparation of any preclinical animal investigation, regulatory agencies (FDA, EMEA, etc.) or local animal welfare agencies must be consulted to determine the welfare and planning of studies prior to initiation. Most countries have their own animal experimental guidelines for animal welfare. Animals must be handled and managed in the most humane environment possible with minimal suffering or loss of life as the goal.

1.1. Common Animal Models Used in Periodontal Research

A variety of different animal models such as rats, dogs, and non-human primates have been used in periodontal regenerative studies (6). For periodontal regeneration studies, the rat periodontal model has been frequently used (7–9). The rat model is quite valuable as a screening tool for regenerative molecule assessment due to cost effectiveness, ease of handling, etc.; however, the typical defect size is relatively small making visualization challenging, thus requiring the use of surgical microscopes for defect creation. Large animal models, such as the canine or the non-human primate, make a logical next step. The canine wound healing kinetics and tooth anatomy have many similarities to the human situation. Non-human primates are highly desirable to evaluate the safety and efficacy of new molecules because their anatomic and biologic features are very close to humans. However, their high cost and the strict regulations needed prevent them from being more highly utilized. One should choose the preferred animal model according to study requirements. Here we review current periodontal regenerative animal models with a focus on rats and canines with specific details on the methods required to perform surgery and evaluate outcome measures.

2. Materials

2.1. Animal Welfare Guidelines

Animal-based research has led to significant improvements in the quality of life for every human being; however, these advances must be the result of humane use and care of animals used for research and instruction. Every investigator should adhere to the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporating principles from the Guide for the Care and Use of Laboratory Animals while executing any work with vertebrate animals. Individual institutions have specific guidelines utilizing policies put in place by the federal and state governments, and it is necessary to review these guidelines

before initiating any effort in animal research. Proper maintenance of documentation and medical and surgical records is also an essential component of compliance with federal and institutional guidelines (*see* **Note 1**).

2.2. Surgical Model

Before performing any in vivo regenerative experiment, the specific animal model should be selected based on outcome. Some animal models may provide critical-sized defects, while others spontaneously heal and are considered as kinetic defects. Thus, it is very important to choose the appropriate model to effectively analyze the effects of bioactive molecules in a specific study. Rat, canine, and non-human primate models are most often used for these experiments. This chapter provides specific details pertaining to the most common periodontal regeneration models (rats and dogs) (*see* **Notes 2 and 3**).

2.2.1. Rat Periodontal Regeneration Model

1. **Figure 18.1** illustrates a periodontal fenestration defect model in the rat.
2. For the rat periodontal fenestration defect (7–9), an extraoral buccal approach should be used. This model is widely employed and is accepted as an appropriate one to test periodontal regeneration in small animals. This model

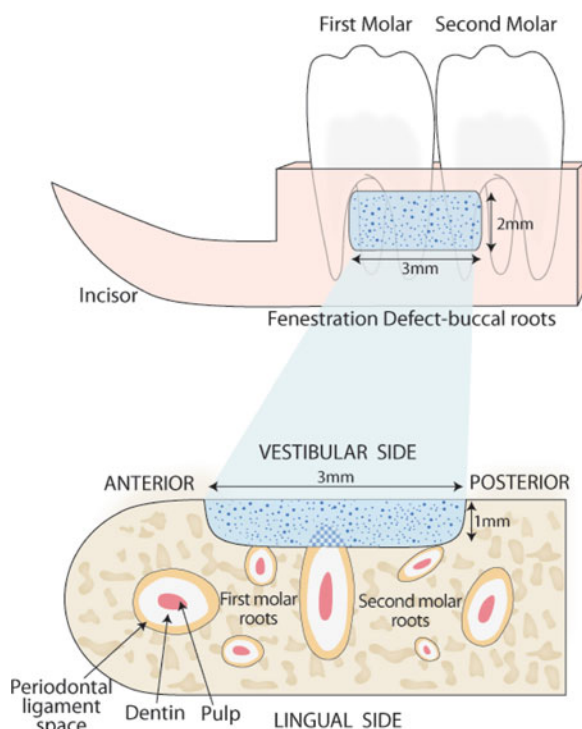


Fig. 18.1. Illustration of rat periodontal defect model. (Reproduced under permission from Pellegrini et al. (6).

provides isolation from the oral environment, due to the extraoral approach, and thus can prevent negative effects such as contamination and infection by saliva and other microorganisms.

2.2.2. Canine Periodontal Regeneration Model

1. **Figure 18.2** illustrates a periodontal surgical model in the canine.
2. The dog is very commonly used as a large animal for periodontal research because it provides many similarities to humans, such as microbiological and periodontal pathogenesis characterization. Premolar teeth have two roots, allowing for creation of critical size, supra-alveolar furcation defects. Furthermore, dogs are generally friendly and cooperative, making care and management easy (10).

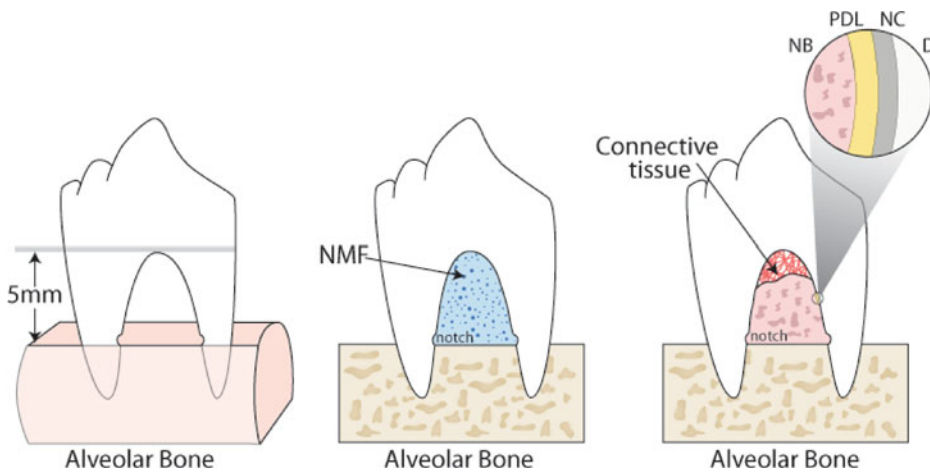


Fig. 18.2. Illustration of dog periodontal defect model. MMF, new medical formulations; NB, new bone; PDL, periodontal ligament; NC, new cementum; and D, dentin. (Reproduced under permission from Pellegrini et al. (6).

2.3. Delivering Devices/Biomaterials

1. **Table 18.1** shows popular delivery vehicles including collagen and hydroxyapatite (*see Note 4*).
2. Bioactive molecules can also be delivered as polypeptides (11, 12), proteins (7, 13), in engineered cells (14), and/or gene vehicles (8).

2.3.1. Viral Vectors as a Delivery Vehicle of Biologic Factors

Gene therapy generally refers to the process of transferring specific genetic sequences to host cells using viral or non-viral vectors in order to produce proteins, with the preferred strategy dependent upon the characteristics of the target site. Viral vectors are more commonly used in current preclinical studies based on their transduction efficiency (15).

1. Adenovirus, adeno-associated virus (AAV), and retrovirus are widely used due to their high infection efficiencies.

Table 18.1**Materials for periodontal/craniofacial repair (reproduced with permission from Anusaksathien et al. (52))**

Biomaterial	Trade name
<i>Allografts</i>	
Calcified freeze-dried bone, decalcified freeze-dried bone	Grafton [®] , Lifenet [®] , Musculoskeletal transplant Foundation [®]
<i>Xenografts</i>	
Bovine mineral matrix, bovine-derived HA	Bio-Oss [®] , OsteoGraf [®] , Pep-Gen P-15 [®]
<i>Alloplasts</i>	
Hydroxyapatite dense HA, porous HA, resorbable HA	
Tricalcium phosphate (TCP), calcium phosphate cement	Synthograft [®] , α -BSM [®]
Hard tissue replacement polymers	Bioplant [®]
Bioactive glass (SiO ₂ , CaO, Na ₂ O, P ₂ O ₅)	PerioGlas [®] , BioGran [®]
Coral-derived calcium carbonate	Biocoral [®]
Polymers and collagens	
Collagen	Helistat [®] , Collacote [®] , Colla-Tec [®] , Gelfoam [®]
Poly(lactide- <i>co</i> -polyglycolide), PLGA	
Methylcellulose	
Hyaluronic acid ester	Hy [®]
Chitosan	
<i>Growth factor</i>	
Platelet-derived growth factor (PDGF)	
Enamel matrix derivative (EMD)	Emdogain [®]
Bone morphogenetic protein (BMP)	
Fibroblast growth factor	
Insulin-like growth factor	
<i>Growth factor + scaffolding material</i>	
Collagen+BMP-2	Infuse [®]
β -TCP+PDGF-BB	GEM21S TM

2. Adenovirus delivers the genetic material in the form of double-stranded DNA and exhibits transiently high transduction efficiency on both dividing and non-dividing cells. The delivered genetic material neither incorporates into the host genome nor induces apparent phenotype change of the infected cells (16).
3. AAV delivers the genetic material in the form of single-stranded DNA and is capable of infecting both dividing and non-dividing cells at a sustained, therapeutic-relevant concentration. Wild-type AAV can insert the genetic material at

a specific site; however, recombinant AAV will not incorporate genetic material into the host genome (17). AAV causes lower immunogenicity than does adenovirus (18).

4. Retrovirus reverse transcribes the RNA genome into the viral DNA and stably integrates into the host genome, resulting in long-term and high-level expression of a transgene. However, retrovirus application may have limited clinical effects due to random incorporation into the host genome as well as infecting only actively dividing cells, making certain types of cells refractory to viral infection (19).

2.4. Biologic Factors

1. Among several growth factors, platelet-derived growth factor (PDGF) (20, 21), fibroblast growth factor-2 (FGF-2) (22, 23), amelogenin (24, 25), and bone morphogenetic proteins (BMPs) (7, 13, 26) are well known to have periodontal regeneration potential (*see* Table 18.1).
2. Combined use of growth factors and scaffolding material may maximize delivery efficiency (27).
3. Combinatory therapy using two (or more) growth factors can also be studied (20, 28).

2.5. Instrumentation

1. Initial incision: Surgical blade (#11, 15), periosteal elevator (Pritchard) (*see* Note 5).
2. Defect creation: Bur, low-speed, and high-speed handpiece with engine and chisel.
3. Small, sharp, hand instrument such as Gracey curette.
4. Wound closure: Needle holder (Crile-Wood), suture material (vicryl resorbable), scissors (LaGrange double curved), and metal staples.
5. Anesthesia; will vary with animal model.
6. Sterile, sanitizable, surgical area with proper ventilation.
7. External heat source(s).
8. Hot-bead instrument sterilizer.
9. Sterile, clean cages for post-surgery animal recovery.
10. Staple remover.
11. Tissue harvesting: Scissors, round disc, and low-speed engine for tissue harvesting.
12. Visualizing microscope. We use a SMZ 1000 (Nikon, Melville, NY).

2.6. Tissue Processing and Embedding

1. Fixation solution:
 - (a) 10% Neutral buffered formalin.
 - (b) 50–70% Ethanol.

2. Tissue processor (Autotechnicon; International Medical Equipment, Inc., San Marcos, CA).
3. Embedding material for decalcified sample preparation: paraffin and paraffin oven.
4. Embedding material for undecalcified sample preparation:
 - (a) Epoxy resin: EMbed 812 (Electronic Microscopic Sciences, Hatfield, PA) or another substitute of EPON812 (29).
 - (b) PMMA (polymethylmethacrylate): Technovit 9100 NEW® (Electronic Microscopic Sciences, Hatfield, PA) or equivalent product.
 - (c) Propylene oxide (Fisher Scientific Co., Pittsburgh, PA) and acetone (Fisher) are also required for epoxy resin and PMMA embedding process.
5. Cutting device for decalcified sample preparation: Microtome.
6. Cutting and grinding device for undecalcified sample preparation:
 - (a) ISOMETRIC diamond saw blade.
 - (b) EXAKT cutting and grinding system.
7. Plastic or glass slides.
8. Ethanol series (70, 80, 90, 95, and 100%) and xylene.
9. Mounting solution – Permount (Fisher).

2.7. Tissue Staining

2.7.1. Hematoxylin and Eosin Stain

1. Gill's hematoxylin 1 (Fisher).
2. Eosin-phloxine B (Fisher).

2.7.2. Methylene Blue Staining

1. Toluidine blue solution: 1 g Toluidine blue and 1 g sodium borate added to 100 mL distilled water and filtered.
2. Toluidine blue equivalent solution: Sanderson's™ Rapid Bone Stain (Surgipath) solution.

2.7.3. Modified Goldner's Masson Staining

1. Weigert's iron hematoxylin.
2. Ponceau-fuchsin solution: 0.75 g Ponceau de xylinine, 0.25 g acid fuchsin, and 1 mL acetic acid added to distilled water to achieve the final volume of 100 mL.
3. Azophloxine solution: 0.5 g azophloxine and 0.6 mL acetic acid added to distilled water to achieve the final volume of 100 mL.
4. Light green solution: 1 g Light green and 1 mL acetic acid added to distilled water to achieve the final volume of 100 mL.

5. Phosphomolybdic acid–orange G solution: 3 g Phosphomolybdic acid and 2 g orange G dissolved in 500 mL distilled water and then added a crystal of thymol.

6. 1% Acetic acid.

2.7.4. von Kossa Stain

1. 1% Silver nitrate (preserve in dark).

2. 2.5% Sodium thiosulfate.

3. 1% Safranin O.

2.7.5. Immunohistochemical Stains

1. 10 mM Sodium citrate (pH~6).

2. 50 mM Tris–HCl buffer.

3. 3% H₂O₂ (in 50 mM Tris–HCl).

4. 1% Bovine serum.

5. Primary and secondary antibody.

6. 3,3'-Diaminobenzidine (DAB) staining solution (DAKO Corp.).

7. Gill's hematoxylin 1.

2.8. Analysis

1. Optical microscope with imaging analysis apparatus. A Nikon Eclipse E800 microscope fitted with a SPOT-2 camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and Image Pro Plus software (Media Cybernetics, Silver Spring, MD) can be used.

2. Micro-CT for additional three-dimensional analysis. eXplore Locus SP and MicroView v.2.0 (Analysis Plus, GE Healthcare) can be used.

3. Methods

3.1. Rat Model

3.1.1. Pre-operative Surgical Preparations

Animals require an acclimation period of approximately 3 days to 1 week after arrival in a new housing facility. The surgical area should be conducive to an aseptic surgery and must not be used for any other purpose during the time of the surgery. Ideally, the surgical area can be located within the housing facilities, therefore limiting stress and potential health hazards to the animals. Disinfectants such as Sporidicin[®], Clorox[®], Clidox[®], Cide-Swipes[®], Virex[®], and Cidex[®] can be used to clean and disinfect the surgery area, although some may not be as effective at eliminating all contaminants.

Animals and instruments must also be prepared in a way to prevent contamination and ensure success of the survival surgery

(*see* **Notes 6** and **7**). Surgeons should also undergo appropriate preparation including, but not limited to, hand washing, wearing sterile gloves, gowns, and masks for each animal's surgical procedures.

3.1.2. Anesthesia

Rats can be anesthetized under general anesthesia by ketamine and xylazine combination via intraperitoneal (IP) injection, lasting for 45–90 min (*see* **Note 8**). To prolong anesthesia, supplement with one-third dose of ketamine only (Horton, Hugunin, Cotroneo). All animals should be provided an external heat source (e.g., recirculating water blanket, microwaveable heating packs, or self-regulating heating pad) in indirect contact with the animal to prevent hypothermia during the entire anesthesia and recovery period.

3.1.3. Surgery

After preparing the animal for surgery, a scalpel incision can be made from skin to the masseter muscle to expose the rat mandibular bone (buccal plate) (*see* **Note 9**). The target area is the distal root of the mandibular first molar. Buccal roots of the first and second molars can be included in the surgical defect (*see* **Fig. 18.1**). Using a round bur with high-speed instrumentation, one can create a bony defect of 3 mm × 2 mm × 1 mm size (*see* **Note 10**). The periodontal ligament, cementum, and superficial dentin can be removed by hand instrumentation. After applying test agent(s) into the created defect area, the muscle and skin are repositioned by sutures and/or surgical clips.

3.1.4. Post-surgical Management

Following surgery, rats may be administered analgesia for pain, and antibiotics for infection control within the surgical site. Analgesics such as buprenorphine (0.01–0.05 mg/kg subcutaneous or intraperitoneal) should be administered for at least 24 h after the periodontal defect surgery. Antibiotics can be dispensed via the water supply (*see* **Note 11**), although normal chow should be readily available (*see* **Note 12**). Animals should be treated and monitored according to the animal surgery guidelines given by their institution in accordance with regulations and in compliance with animal housing authorities. For example, if biohazardous materials, such as viral vectors, are applied, the animal must be kept in biohazard facility until viral shedding has completed (*see* **Note 13**).

3.1.5. Sacrifice and Harvesting

At study endpoints, animals can be sacrificed and tissues harvested for analysis. Experimental endpoints are determined by previous studies, published literature, and/or may be based on the biological material properties themselves. If the molecules of interest act early in the healing process, the endpoints should be selected accordingly in order to capture important healing events. Euthanasia methods, such as CO₂ asphyxiation, should

be selected according to institutional guidelines. A secondary method should be employed in order to confirm animal death prior to resuming tissue collection. Harvested samples should be fixed to prevent degradation without damaging the tissues.

3.2. Canine Model

3.2.1. Pre-operative Surgical Preparations

1. Animals require an acclimation period of approximately 3 days to 1 week after arrival in a new housing facility. The surgical area should be easily sanitizable and undergo careful preparation to ensure that aseptic surgical technique is carried out according to government and institutional guidelines. Ideally, the surgical area can be located within the housing facilities but should be separated from human occupancy areas, therefore limiting stress and potential health hazards to the animals. Disinfectants such as Sporocidin[®], Clorox[®], Clidox[®], Cide-Swipes[®], Virex[®], and Cidex[®] can be used to clean and disinfect the surgery area, although some may not be effective at eliminating all contaminants.
2. Animals and instruments must also be prepared in a way to limit or prevent contamination and ensure success of the survival surgery (*see* **Notes 7 and 14**). Surgeons should undergo appropriate preparation in a room separate from the operating areas, including, but not limited to surgical staff, wearing sterile gloves, gowns, caps, shoe covers, and masks for each animal's surgical procedures.
3. The pre-surgical oral hygiene phase is especially necessary for canine and non-human primates to obtain a healing response following surgery by ensuring biofilm removal and to begin with healthy periodontal tissues. Scaling and root planing procedures should be performed in an area separate from the surgical operating room to prevent contamination and can be carried out approximately 10 days prior to surgery.

3.2.2. Anesthesia

Prior to anesthesia, canines greater than 10 weeks of age should be fasted for at least 6 h, with the exception of water. Canines should be sedated using a combination of buprenorphine, acepromazine, and glycopyrrolate as a pre-sedative approximately 30–60 min prior to propofol administration for induction (*see* **Note 15**). The canine will then be intubated and maintained under general anesthesia with isoflurane delivered through a volume-regulated aspirator (**13**). Intravenous fluids, such as Ringer's solution (10 mL/kg/h), should be administered during surgery (*see* **Note 16**). Local infiltration anesthesia, for example, lidocaine HCl with epinephrine is helpful to limit bleeding at and near to the surgical site. All animals should be provided

an external heat source, preferably a recirculating water blanket, to prevent hypothermia during the entire anesthesia and recovery period.

3.2.3. Surgery

1. *Defect creation*: Periodontal defects can either be created surgically (13, 30, 31), involve ligatures around teeth (32, 33), or develop naturally (34, 35). For surgical creation, defects can be made to the same size or shape to compare test groups and controls. The defects are typically created around the second, third, and fourth mandibular premolar teeth. After sulcular incisions and mucoperiosteal flap elevations, alveolar bone is removed from around the teeth to make a circumferential defect using chisels and burs with saline irrigation. Resection can be restricted to the inter-root area which measures ~4 mm in height and 3 mm in width (36–38) or extended to create a horizontal circumferential defect up to 5 mm below the fornix of the furcation (13, 39, 40) (Fig. 18.2). Some protocols elect to extract the first mandibular premolar followed by amputation of the first molar level with the crest of the surgically reduced alveolar bone (30). After resection, bone and cementum are removed, and notches are created to mark the lowest point of exposed roots in order to make a distinction between the old and new bone levels during evaluation.
2. *Regenerative treatment*: In the acute defect model, lesions are immediately treated with test agents. In the chronic defect model, the root surface is exposed to the oral environment in order to minimize spontaneous tissue regeneration (30). Regenerative surgery can be performed after 4–6 months (30, 41) of healing in combination with scaling and root planing via open flap surgery. Regenerative molecules are then applied around the defect area and flaps are closed.

3.2.4. Post-surgical Management

1. *Pain and infection control*: Animals should be monitored until alert and active, and adequate homeostasis is achieved. While the animal is semi-conscious, it should be monitored every 15 min and then monitored every hour until fully conscious. Once conscious, the animal must be monitored twice daily until fully recovered from the surgical procedure. Recovery should be carried out in a quiet, temperature-controlled, designated recovery area separate from the normal cage. Administration of carprofen every 12–24 h and/or buprenorphine every 8–12 h can be carried out for post-surgery pain relief (*see Note 17*). Antibiotics, such as penicillin G benzathine (50,000 U/kg) should be administered subcutaneously on the day of surgery and every 5 days for up to 2 weeks.

2. *Oral hygiene maintenance*: For hygiene control, the treatment sites are to be cleaned with 0.1–0.2% chlorhexidine gel for 3 weeks following surgery. After the 3-week period, canines will receive a supragingival debridement and prophylaxis every 2 weeks. Every effort should be made to maintain optimal oral hygiene for each animal. Acepromazine (0.03–0.25 mg/kg i.m., i.v., or s.c.) may be used as a tranquilizer prior to oral hygiene measures, if necessary.
3. *Diet and miscellaneous*: The animals are to be given a soft chow diet for up to 2 weeks following all surgical procedures to minimize trauma to the periodontium, until dry chow can be tolerated (*see Note 12*). Sutures must be removed between 10 and 14 days post-surgery, under anesthesia if necessary. Surgical records should be kept in the animal housing area for 3–7 days following anesthesia and/or surgery.

3.2.5. Sacrifice and Harvesting

1. *Sacrifice*: At study endpoints, animals can be sacrificed and tissues harvested for analysis. Experimental endpoints are determined based on previous studies, published literature, and/or may be based on the biological material properties themselves. If the molecule of interest acts early in the healing process, endpoints should be selected accordingly in order to capture important healing events involving the compound of interest. Euthanasia methods, such as barbiturate overdose, should be selected according to institutional guidelines. A secondary method should be employed in order to confirm animal death prior to tissue collection. Harvested samples should be immediately fixed to prevent degradation or damage occurring to the tissues.
2. *Harvesting tissue*: For rat jawbone harvesting, a round diamond disk saw are used to cut hard tissues and scissors are used with soft tissues. The entire jaw can be harvested in whole. For canine jaw bone harvesting, a coping saw can be used to cut hard tissues. Each tooth segment can be sectioned by using a diamond saw.

3.3. Histological Sample Preparation

3.3.1. Fixation

1. 10% Formalin: 24–48 h, then replaced by 70% ethanol.
2. Ethanol: At least 72 h.

3.3.2. Decalcification

Use 10% EDTA at 4°C for 2–4 weeks. Alternatively 10% acetic acid, 4% formaldehyde, 0.85% NaCl for 2–3 weeks.

3.3.3. Dehydration and Infiltration Process of Paraffin-Embedded Specimens (In Tissue Processor/Autotechnicon)

1. Ethanol series [70, 80, 95% (twice) and 100% (three times)] for 1 h in each dilution.
2. Xylene for 0.5–1 h (twice).
3. Wax (paraffin) bath for 1.5 h (twice).

3.3.4. Casting for Paraffin-Embedded Specimens

1. Pour melted wax (use paraffin oven) to enclose specimen on the metal plate.

3.3.5. Sectioning for Paraffin-Embedded Specimens

1. Use a microtome to achieve a final thickness of 4–8 μm for glass slide mounting.

3.3.6. Dehydration and Infiltration of Epoxy Resin Specimens

1. Ethanol series [70, 80, 90, and 100% (twice)] for 1 h in each dilution.
2. Acetone for 2 h.
3. Propylene oxide for 15 min (twice).
4. 1:1 EPON 812/propylene oxide for 1.5 h.
5. 2:1 EPON 812/propylene oxide overnight.
6. EPON 812 for 30 min to 2 h at room temperature.
7. EPON 812 for 16–24 h in 55–65°C.

3.3.7. Dehydration and Infiltration of PMMA Specimens

1. Ethanol series [70, 80, 90, 95, and 100% (twice)] for 12 h in each dilution at 4°C.
2. Xylene for 12 h at 4°C (twice).
3. 1:1 PMMA/xylene for 24 h at 4°C.
4. PMMA and 0.5% hardener for 24 h at 4°C.
5. PMMA infiltrate for 2–3 days at –20°C.

3.3.8. Sectioning for Plastic-Embedded Specimens

1. Use EXAKT cutting device or ISOMETRIC diamond saw blade to achieve the final thickness of around 200 μm .
2. Mount on the plastic slide using methyl cyanoacrylate glue.
3. Polishing: Use EXAKT grinding system with sandpaper from 800 to 2,400 grits to achieve a final thickness of around 50–100 μm .

3.3.9. Hematoxylin and Eosin Stain (For Paraffin-Embedded Specimens)

1. Immerse in xylene (Fisher) for 3–5 min (*see Table 18.2*).
2. Rinse in gradient concentration of ethanol (70, 80, 90, and 100%).
3. Gill's hematoxylin 1 for 2 min.
4. Rinse with water.

Table 18.2
Staining methods and typical staining characteristics for each cellular component or tissue

Objects	Hematoxylin and eosin stain	Methylene blue stain	Modified Goldner's Masson stain	von Kossa stain	Immunohisto-chemical stain
Nuclei	Blue/black	Blue/black	Blue-gray		
Cytoplasm	Pink	Pink			
Bone	Pink	Pink			
Osteoid	Pink	Pink	Orange-red	Red	
Fibers	Deep pink/red	Deep pink/red		Red	
Mineralized tissue			Green	Gold/Brown	
Cartilage			Purple		
Antigens					Brown
Background					Pink/blue

5. Eosin-phloxine B for 1 min.
6. Rinse in gradient concentration of ethanol (100, 90, 80, and 70%).
7. Xylene for at least 3 min.

3.3.10. Methylene Blue Staining (For Epoxy Resin- or PMMA-Embedded Specimens)

1. Remove embedding material (*see* **Table 18.2**) (42).
2. 0.1% Formic acid (Fisher) for 1–2 min.
3. Rinse with water.
4. 95% Ethanol for 2 min.
5. 100% Ethanol for 2 min.
6. Xylene-float on the surface for 6–8 min.
7. 100, 95, and 70% Ethanol for 2 min in each.
8. Toluidine blue solution at 37°C for 4–5 min or Sanderson'sTM Rapid Bone Stain for 10–12 min.
9. Rinse in distilled water.
10. Counter stain by acid fuchsin (Sigma).

3.3.11. Modified Goldner's Masson Staining

1. Remove the embedding material on the surface of the specimen (43) (*see* **Table 18.2**).
2. Alkaline alcohol solution (90 mL of 80% ethanol, 10 mL of 25% ammonia) for 1 h.
3. Rinse with water for 15 min.

4. Weigert's iron hematoxylin for 1 h.
5. Rinse with water for 10 min.
6. Rinse in distilled water for 5 min.
7. Ponceau–fuchsin–azophloxine solution (5–10 mL Ponceau–fuchsin, 2 mL azophloxine, and 88 mL of 0.2% acetic acid) for 5 min.
8. Rinse in 1% acetic acid for 15 s.
9. Phosphomolybdic acid–orange G solution for 20 min.
10. Rinse in 1% acetic acid for 15 s.
11. Light green solution for 5 min.
12. Rinse in 1% acetic acid for 15 s (three times).
13. Rinse in distilled water.

3.3.12. von Kossa Staining

1. Remove the embedding material on the surface of the specimen (27) (for undecalcified sections only, *see* Table 18.2).
2. Silver nitrate exposed to strong light for 30–60 min.
3. Rinse with water (three times).
4. Sodium thiosulfate for 5 min.
5. Rinse with water.
6. Counter stain by safranin O.

3.3.13. Immunohistochemical Stains

1. Remove the embedding material on the surface of specimen (8, 27) (*see* Table 18.2).
2. 10 mM Sodium citrate for 10 min at 90–95°C.
3. Cool to room temperature, rinse with 50 mM Tris–HCl twice.
4. Block with 3% H₂O₂ in 50 mM Tris–HCl for 5 min.
5. Wash and rinse with water.
6. 1:50~1:1,000 dilution of primary antibody in 50 mM Tris–HCl and 1% bovine serum for 10 min to 2 h (varies with manufacturer's instructions).
7. Wash and rinse with water.
8. 1:50~1:1,000 dilution of secondary antibody for 10 min to 2 h (varies with manufacturer's instructions).
9. Wash and rinse with water.
10. 3,3'-Diaminobenzidine (DAB) staining solution for 3–5 min.
11. Counter stain by Gill's hematoxylin 1.
12. Mount by permount and cover with glass cover.

3.4. Result Evaluation

If the study involves evaluation of the safety of new bioactive molecules, the following examinations are suggested:

1. Clinical observation – Changes in body weight, local swelling, edema, and/or inflammation of the surgical area.
2. Hematology – Complete blood cell and platelet counts, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and red blood cell distribution width measurements.
3. Clinical chemical parameters (from serum) – Albumin, alkaline phosphatase, alanine transaminase, amylase, aspartate transaminase, bilirubin, calcium, cholesterol, creatine kinase, creatinine, glucose, phosphorus, total bilirubin, total protein, and globulin levels.
4. Major organ examinations – Surgical site and local tissues, salivary glands, local lymph node drainage, brain, heart, lung, liver, spleen, kidney, and gonads (testes or ovaries).

3.5. Descriptive Histology/ Histomorphometry

3.5.1. Descriptive Histology

For studies involving tissue regeneration, histological images provide basic evidence of the effect of molecules of interest on cells and tissues. Therefore, it is indispensable to clearly identify and display the histological results. Every detail must be described in order to understand and explain the findings, especially to identify tissues affected, including but not limited to the affected tissue boundaries, location, collagen fiber alignment, cell characteristics, and tissue maturation.

3.5.2. Histomorphometry of the Rat Model

The following criteria can be measured for histomorphometry analysis:

1. Length of new cementum
2. Length of new bone
3. Length of alveolar bone fill (8)

3.5.3. Histomorphometry of Canine Periodontal Regeneration Model

The following criteria can be measured for histomorphometry analysis (44, 45):

1. Area of new bone.
2. Length of new cementum (*see Note 18*).
3. Length of complete new attachment apparatus (CNAA).
4. Percentage of defect filled with new bone and new cementum.

5. Length of crestal bone resorption/formation.
6. Length of apical migration of functional epithelium.
7. New bone formation can be further measured (46) with the following criteria: bone surface/perimeter, bone volume/area, tissue volume/area, core volume/area, osteoid surface/perimeter, eroded surface/perimeter, mineralizing surface/perimeter, osteoblast surface/perimeter, and osteoclast surface/perimeter.

3.6. Microcomputed Tomography (μ CT)

Conventional histological examination or histomorphometry provides direct representations of alveolar bone levels and histopathological phenomena with high resolution. In addition, these methods can analyze soft tissues, lamellar or woven bone quality, or external circumferential lamellae. However, this destructive method has a limitation to quantify whole regenerated periodontal tissue. In the case of analytical quantification and anatomical visualization of the mineralized tissue structures, micro-CT imaging is currently highlighted in medicine with a series of polychromatic radiographic images with micrometer-level voxel size. Micro-CT can provide a more accurate general profile and more detailed information in 3D structure (47).

3.6.1. Advantages of Micro-CT

1. Provides 3D assessments of whole and regenerated osseous tissues.
2. Provides volumetric mineralized tissue measurement (*see Note 19*).
3. Bone density can be calculated.
4. Degree of mineralization can be calculated.

3.6.2. Disadvantages of Micro-CT

1. Difficulty in identifying borders of newly mineralized tissue from old mineralized tissue.
2. Limited information of soft tissue, such as range of junctional epithelium.
3. Limited information regarding cellular level histology.
4. If the specimen contains metallic material, image scattering may occur around it.

4. Notes

1. Notes and records of the entire surgical and post-surgical processes are critical in ensuring proper animal care and lead to greater understanding of experimental results. Records should include, but are not limited to, animal identification, date/time of surgery, pre-surgical body

weight, dose and route of anesthesia, period of anesthesia, surgical treatment, length of surgery, recovery period, surgical outcome, manner of disposition, and USDA identification number (canines only). Documentation of 5–7 days post-operative monitoring should be kept for all rodent procedures. Documentation of canines' post-anesthesia and post-surgical medical records should be kept for 3 years following the completion of the study.

2. *Animal selection considerations.* Generally, in vivo experiments should move from small to large animals. Small animal models cost less and are generally easier to house, access, and work with. Upon completion and confirmation of small animal testing, large animal models can be used as they are physiologically more similar to humans, and experimental results will better translate to humans for potential clinical research. Specifically relating to oral surgical models, canines were historically chosen for their similarity in histological arrangement of canine periodontium and dentogingival attachment to that of humans.
3. *Strain, age, and gender.* The strain and age of the animal should be considered in order to achieve experimental objectives and for comparison to other studies. In young rats (7), periodontal regeneration is complete after 1 month, while geriatric animals at 18 months of age show a delayed healing capacity (48). It is therefore crucial to select the appropriate time point(s) to determine the therapeutic efficacy of a bioactive molecule. It should also be considered that as animals age, they will have decreased life expectancies and may develop systemic complications that are not externally visible. The specific animal strain is critical to understanding the animals' response to the surgical procedure and treatment. Gender can also affect the results, especially if the animals are osteoporotic, and viral transduction, for example, may be different according to the gender (49). Previous experiments, vendor information, and published literature should be used in determining the details of the selected animal model.
4. Treatment delivery vehicle should be chosen based on previous experiments and published literature. Delivery material should be biocompatible and easy to handle within the confines of the experiment. It is necessary to test the release kinetics of test articles from the candidate delivery material prior to animal experiment initiation.
5. *Surgical instrumentation.* The periosteal elevator will be used for flap elevation, the Gracey curette for root planing, and the bur in handpiece with engine and chisel for defect creation.

6. *Animal preparation for rodent surgery.* Following anesthesia, ophthalmic ointment (e.g., Puralube[®]) must be applied to the eyes of the animal receiving anesthetic to prevent drying. Incision sites must be cleared of hair if the incision is >1 cm, using clippers, a razor, or depilatory agent. Hair removal should be performed in a separate location so as to avoid surgical area contamination. Skin must then be disinfected with three alternating scrubs of iodophor (e.g., Betadine[®]) and warm, sterile saline, water, or 70% ethanol (ethanol is less desirable), scrubbing in an outward and spiral direction.
7. All instruments should be cleaned and sterilized (e.g., autoclaved) prior to surgery. Disinfection/sterilization of multiple sets of instruments should be carried out for multiple surgeries. Following use, instruments should be thoroughly cleaned before sterilization. Hot-bead sterilization is a fast, dry method to prevent cross-contamination between animals during surgery. Alternative sterilization methods may incorporate the use of Sporocidin[®], chlorine dioxide, or Cidex[®] immersion followed by a sterile water or saline rinse. Aseptic techniques and sterile environments are critical to animal survival and positive experimental results. Effective drug dosage may vary from animal to animal according to body weight, metabolism, and age. There are no exact calculations to relate effective dose between animal and humans. Dosage can be determined by previous study results, published literature, and veterinary guidelines.
8. *Rat anesthetics and analgesics.* A combination of ketamine (i.p., 40–90 mg/kg) and xylazine (i.p., 5–10 mg/kg) can be used as a general, injectable anesthesia for oral procedures. To prolong anesthesia, supplement with one-third dose of ketamine only. IP injections should be performed using a 20–27-gauge needle that is inserted into the lower left abdominal quadrant with the animal in a head-down position. Buprenorphine (subcutaneous, 0.01–0.05 mg/kg) can be used for 8–12 h for post-operative pain relief or 5 mg/kg ketoprofen (subcutaneous) may be selected for 24-h pain management. Anesthesia depth is typically monitored by the loss of response to external stimuli, such as a limb pinch.
9. In some cases, the parotid gland duct (Stenson's) can be involved, causing post-surgical buccal swelling. This swelling can affect tissue regeneration as it produces mechanical pressure to the surgical area. Swelling can be eliminated by surgical removal of the parotid gland in those rare cases.

10. During bone and cementum removal, it is difficult to irrigate with saline due to the small defect size, thin bone, and cementum. Special care should be taken not to generate heat damage at the surgical site, as it prevents a normal healing process.
11. Antibiotics may be added to the drinking water in order to reduce the incidence of infection following oral surgery. Ampicillin (268 mg/L) may be added to a 5–10% dextrose solution. Colored water bottles should be used with light-sensitive antibiotics.
12. Animals should be mobile and feed freely following surgical recovery. Animal recovery time will vary with the type of anesthesia and dose of anesthesia, and may also vary between animals of similar sex, size, body mass, and genetic background. During the recovery process, all animals should be housed individually.
13. If viral vectors are used, each virus will have a specific shedding period for which it can be considered contagious, and contact should be limited during this period. For example, adenovirus should be considered biohazardous for at least 72 h following application/inoculation.
14. *Animal preparation for surgery – canine.* Preparation must be carried out in a room separate from the actual operating areas. Following pre-anesthesia administration, prior to induction, ophthalmic ointment must be applied to the eyes of the animal to prevent drying. Hair removal from the surgical site will be performed in a separate location so as not to contaminate the surgical area. Sites will then be disinfected with three alternating scrubs of iodophor (e.g., Betadine®) and warm, sterile saline, or water, scrubbing in an outward spiral direction. Cephalic catheter can be used for ease of i.v. administration of fluids and drugs.
15. *Canine anesthetics.* BAG (i.v. combination of 0.01–0.02 mg/kg buprenorphine, 0.03–0.05 mg/kg acepromazine, and 0.01 mg/kg glycopyrrolate) can be used as a sedative, 3–6 mg/kg of propofol (i.v.) can be given to effect for anesthesia induction/intubation, and 1–2% isoflurane (inhalation) can be used to maintain general anesthesia during surgery. Atropine may be utilized as needed during canine surgery for the treatment of bradycardia while the animal is under general anesthesia. Local pain relief may be relieved by 1–2 mg/kg bupivacaine. Anesthesia depth can be monitored by loss of blink reflexes, jaw tone (tense = inadequate anesthesia), heart rate, and respiratory rate (60–80 beats per minute, 10–12 breaths per minute with anesthesia).

16. If blood loss does occur during surgery, three times the estimated volume of blood loss should be administered via intravenous fluids.
17. *Canine analgesics*. Carprofen (2–4.4 mg/kg, subcutaneous) and/or buprenorphine (0.01–0.02 mg/kg, subcutaneous) can be used for post-operative pain relief.
18. The process of regeneration must be considered when determining time of sacrifice. Canine cementum formation (50, 51) on root planed surfaces occurs after approximately 3 weeks. Considerable amounts of new cementum and new bone filling the furcations may form after 6 weeks. The use of bioactive molecules may accelerate the healing process; thus preliminary studies should be completed with specific test articles to determine ideal evaluation time points in pilot studies.
19. The 3D volume is calculated by measuring the area in a slice-by-slice manner. After interpolating the region of interest and generating 3D constructs, bone parameters can be calculated. The measurements can be affected by the frame selected. Thus, natural anatomy such as distal and mesial roots can be used as landmarks for choosing the frame which can produce reliable and reproducible results (47).

References

1. Ma, P. X., Schloo, B., Mooney, D., and Langer, R. (1995) Development of biomechanical properties and morphogenesis of in vitro tissue engineered cartilage. *J. Biomed. Mater. Res.* **29**, 1587–1595.
2. Jin, Q., Wei, G., Lin, Z., Sugai, J. V., Lynch, S. E., Ma, P. X., and Giannobile, W. V. (2008) Nanofibrous scaffolds incorporating PDGF-BB microspheres induce chemokine expression and tissue neogenesis in vivo. *PLoS ONE*. **3**, e1729.
3. Van de Putte, K. A., and Urist, M. R. (1965) Osteogenesis in the interior of intramuscular implants of decalcified bone matrix. *Clin. Orthop. Relat. Res.* **43**, 257–270.
4. Antoniadis, H. N., Scher, C. D., and Stiles, C. D. (1979) Purification of human platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA*. **76**, 1809–1813.
5. Sternfeld, M. D., Hendrickson, J. E., Keeble, W. W., Rosenbaum, J. T., Robertson, J. E., Pittelkow, M. R., and Shipley, G. D. (1988) Differential expression of mRNA coding for heparin-binding growth factor type 2 in human cells. *J. Cell. Physiol.* **136**, 297–304.
6. Pellegrini, G., Seol, Y. J., Gruber, R., and Giannobile, W. V. (2009) Preclinical models for oral and periodontal reconstructive therapies. *J. Dent. Res.* **88**, 1065–1076.
7. King, G. N., King, N., Cruchley, A. T., Wozney, J. M., and Hughes, F. J. (1997) Recombinant human bone morphogenetic protein-2 promotes wound healing in rat periodontal fenestration defects. *J. Dent. Res.* **76**, 1460–1470.
8. Jin, Q., Anusaksathien, O., Webb, S. A., Printz, M. A., and Giannobile, W. V. (2004) Engineering of tooth-supporting structures by delivery of PDGF gene therapy vectors. *Mol. Ther.* **9**, 519–526.
9. Huang, K. K., Shen, C., Chiang, C. Y., Hsieh, Y. D., and Fu, E. (2005) Effects of bone morphogenetic protein-6 on periodontal wound healing in a fenestration defect of rats. *J. Periodontol. Res.* **40**, 1–10.
10. Attstrom, R., Graf-de Beer, M., and Schroeder, H. E. (1975) Clinical and histologic characteristics of normal gingiva in dogs. *J. Periodontol. Res.* **10**, 115–127.
11. Seol, Y. J., Park, Y. J., Lee, S. C., Kim, K. H., Lee, J. Y., Kim, T. I., Lee, Y. M., Ku, Y.,

- Rhyu, I. C., Han, S. B., and Chung, C. P. (2006) Enhanced osteogenic promotion around dental implants with synthetic binding motif mimicking bone morphogenetic protein (BMP)-2. *J. Biomed. Mater. Res. A* **77**, 599–607.
12. Vastardis, S., Yukna, R. A., Mayer, E. T., and Atkinson, B. L. (2005) Periodontal regeneration with peptide-enhanced anorganic bone matrix in particulate and putty form in dogs. *J. Periodontol.* **76**, 1690–1696.
13. Giannobile, W. V., Ryan, S., Shih, M. S., Su, D. L., Kaplan, P. L., and Chan, T. C. (1998) Recombinant human osteogenic protein-1 (OP-1) stimulates periodontal wound healing in class III furcation defects. *J. Periodontol.* **69**, 129–137.
14. Saygin, N. E., Tokiyasu, Y., Giannobile, W. V., and Somerman, M. J. (2000) Growth factors regulate expression of mineral associated genes in cementoblasts. *J. Periodontol.* **71**, 1591–1600.
15. Ramseier, C. A., Abramson, Z. R., Jin, Q., and Giannobile, W. V. (2006) Gene therapeutics for periodontal regenerative medicine. *Dent. Clin. North Am.* **50**, 245–263.
16. Chang, P. C., Cirelli, J. A., Jin, Q., Seol, Y. J., Sugai, J. V., D'Silva, N. J., Danciu, T. E., Chandler, L. A., Sosnowski, B. A., and Giannobile, W. V. (2009) Adenovirus encoding human platelet-derived growth factor-B delivered to alveolar bone defects exhibits safety and biodistribution profiles favorable for clinical use. *Hum. Gene. Ther.* **20**, 486–496.
17. Robbins, P. D., and Ghivizzani, S. C. (1998) Viral vectors for gene therapy. *Pharmacol. Ther.* **80**, 35–47.
18. Cirelli, J. A., Park, C. H., MacKool, K., Taba, M., Jr., Lustig, K. H., Burstein, H., and Giannobile, W. V. (2009) AAV2/1-TNFR:Fc gene delivery prevents periodontal disease progression. *Gene Ther.* **16**, 426–436.
19. Walther, W., and Stein, U. (2000) Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs*. **60**, 249–271.
20. Giannobile, W. V., Hernandez, R. A., Finkelman, R. D., Ryan, S., Kiritsy, C. P., D'Andrea, M., and Lynch, S. E. (1996) Comparative effects of platelet-derived growth factor-BB and insulin-like growth factor-I, individually and in combination, on periodontal regeneration in *Macaca fascicularis*. *J. Periodontol. Res.* **31**, 301–312.
21. Park, J. B., Matsuura, M., Han, K. Y., Norderyd, O., Lin, W. L., Genco, R. J., and Cho, M. I. (1995) Periodontal regeneration in class III furcation defects of beagle dogs using guided tissue regenerative therapy with platelet-derived growth factor. *J. Periodontol.* **66**, 462–477.
22. Shimabukuro, Y., Ichikawa, T., Takayama, S., Yamada, S., Takedachi, M., Terakura, M., Hashikawa, T., and Murakami, S. (2005) Fibroblast growth factor-2 regulates the synthesis of hyaluronan by human periodontal ligament cells. *J. Cell Physiol.* **203**, 557–563.
23. Murakami, S., Takayama, S., Ikezawa, K., Shimabukuro, Y., Kitamura, M., Nozaki, T., Terashima, A., Asano, T., and Okada, H. (1999) Regeneration of periodontal tissues by basic fibroblast growth factor. *J. Periodontol. Res.* **34**, 425–430.
24. Araujo, M. G., and Lindhe, J. (1998) GTR treatment of degree III furcation defects following application of enamel matrix proteins. An experimental study in dogs. *J. Clin. Periodontol.* **25**, 524–530.
25. Bosshardt, D. D. (2008) Biological mediators and periodontal regeneration: a review of enamel matrix proteins at the cellular and molecular levels. *J. Clin. Periodontol.* **35**, 87–105.
26. Saito, E., Saito, A., and Kawanami, M. (2003) Favorable healing following space creation in rhBMP-2-induced periodontal regeneration of horizontal circumferential defects in dogs with experimental periodontitis. *J. Periodontol.* **74**, 1808–1815.
27. Chang, P. C., Liu, B. Y., Liu, C. M., Chou, H. H., Ho, M. H., Liu, H. C., Wang, D. M., and Hou, L. T. (2007) Bone tissue engineering with novel rhBMP2-PLLA composite scaffolds. *J. Biomed. Mater. Res. A* **81**, 771–780.
28. Lynch, S. E., Williams, R. C., Polson, A. M., Howell, T. H., Reddy, M. S., Zappa, U. E., and Antoniades, H. N. (1989) A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration. *J. Clin. Periodontol.* **16**, 545–548.
29. Luft, J. H. (1961) Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**, 409–414.
30. Wikesjo, U. M., Kean, C. J., and Zimmerman, G. J. (1994) Periodontal repair in dogs: supraalveolar defect models for evaluation of safety and efficacy of periodontal reconstructive therapy. *J. Periodontol.* **65**, 1151–1157.
31. Shirakata, Y., Yoshimoto, T., Goto, H., Yonamine, Y., Kadomatsu, H., Miyamoto, M., Nakamura, T., Hayashi, C., and Izumi, Y. (2007) Favorable periodontal healing of 1-wall infrabony defects after application of calcium phosphate cement wall alone or in combination with enamel matrix derivative:

- a pilot study with canine mandibles. *J. Periodontol.* **78**, 889–898.
32. Martuscelli, G., Fiorellini, J. P., Crohin, C. C., and Howell, T. H. (2000) The effect of interleukin-11 on the progression of ligature-induced periodontal disease in the beagle dog. *J. Periodontol.* **71**, 573–578.
 33. Nociti, F. H., Jr., Cesco De Toledo, R., Machado, M. A., Stefani, C. M., Line, S. R., and Goncalves, R. B. (2001) Clinical and microbiological evaluation of ligature-induced peri-implantitis and periodontitis in dogs. *Clin. Oral Implants Res.* **12**, 295–300.
 34. Lekovic, V., Klokkevold, P. R., Kenney, E. B., Dimitrijelic, B., Nedic, M., and Weinlaender, M. (1998) Histologic evaluation of guided tissue regeneration using 4 barrier membranes: a comparative furcation study in dogs. *J. Periodontol.* **69**, 54–61.
 35. Lynch, S. E., de Castilla, G. R., Williams, R. C., Kiritsy, C. P., Howell, T. H., Reddy, M. S., and Antoniadis, H. N. (1991) The effects of short-term application of a combination of platelet-derived and insulin-like growth factors on periodontal wound healing. *J. Periodontol.* **62**, 458–467.
 36. Araujo, M. G., Berglundh, T., and Lindhe, J. (1998) GTR treatment of degree III furcation defects with 2 different resorbable barriers. An experimental study in dogs. *J. Clin. Periodontol.* **25**, 253–259.
 37. Araujo, M. G., Berglundh, T., Albrektsson, T., and Lindhe, J. (1999) Bone formation in furcation defects. An experimental study in the dog. *J. Clin. Periodontol.* **26**, 643–652.
 38. Chen, F. M., Zhao, Y. M., Wu, H., Deng, Z. H., Wang, Q. T., Zhou, W., Liu, Q., Dong, G. Y., Li, K., Wu, Z. F., and Jin, Y. (2006) Enhancement of periodontal tissue regeneration by locally controlled delivery of insulin-like growth factor-I from dextran-co-gelatin microspheres. *J. Control. Release.* **114**, 209–222.
 39. Wikesjo, U. M., Xiropaidis, A. V., Thomson, R. C., Cook, A. D., Selvig, K. A., and Hardwick, W. R. (2003) Periodontal repair in dogs: space-providing ePTFE devices increase rhBMP-2/ACS-induced bone formation. *J. Clin. Periodontol.* **30**, 715–725.
 40. Wikesjo, U. M., and Nilveus, R. (1991) Periodontal repair in dogs. Healing patterns in large circumferential periodontal defects. *J. Clin. Periodontol.* **18**, 49–59.
 41. Morris, M. S., Lee, Y., Lavin, M. T., Gianini, P. J., Schmid, M. J., Marx, D. B., and Reinhardt, R. A. (2008) Injectable simvastatin in periodontal defects and alveolar ridges: pilot studies. *J. Periodontol.* **79**, 1465–1473.
 42. Sterchi, D. L., and Eurell, J. A. (1989) A new method for preparation of undecalcified bone sections. *Stain Technol.* **64**, 201–205.
 43. Kiernan, J. A. (2008) *Histological and histochemical methods: theory and practice*, 4th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
 44. Giannobile, W. V., Finkelman, R. D., and Lynch, S. E. (1994) Comparison of canine and non-human primate animal models for periodontal regenerative therapy: results following a single administration of PDGF/IGF-I. *J. Periodontol.* **65**, 1158–1168.
 45. Lynch, S. E. (1992) Methods for evaluation of regenerative procedures. *J. Periodontol.* **63**, 1085–1092.
 46. Vedi, S., and Compston, J. (2003) *Bone histomorphometry. Bone research protocol*. Human Press, Totowa, NJ, pp. 283–298.
 47. Park, C. H., Abramson, Z. R., Taba, M., Jr., Jin, Q., Chang, J., Kreider, J. M., Goldstein, S. A., and Giannobile, W. V. (2007) Three-dimensional micro-computed tomographic imaging of alveolar bone in experimental bone loss or repair. *J. Periodontol.* **78**, 273–281.
 48. Benatti, B. B., Neto, J. B., Casati, M. Z., Sallum, E. A., Sallum, A. W., and Nociti, F. H., Jr. (2006) Periodontal healing may be affected by aging: a histologic study in rats. *J. Periodontol. Res.* **41**, 329–333.
 49. Davidoff, A. M., Ng, C. Y., Zhou, J., Spence, Y., and Nathwani, A. C. (2003) Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. *Blood.* **102**, 480–488.
 50. Grigger, M., Bogle, G., Nilveus, R., Egelberg, J., and Selvig, K. A. (1978) The effect of topical citric acid application on the healing of experimental furcation defects in dogs. *J. Periodontol. Res.* **13**, 538–549.
 51. Selvig, K. A., Ririe, C. M., Nilveus, R., and Egelberg, J. (1981) Fine structure of new connective tissue attachment following acid treatment of experimental furcation pockets in dogs. *J. Periodontol. Res.* **16**, 123–129.
 52. Anusaksathien, O., Jin, Q. M., Ma, P. X., and Giannobile, W. V. (2006) *Scaffolding in tissue engineering*, Vol. 9. CRC Press, Florida, pp. 437–454.

Chapter 19

Proteomic Analysis of Dental Tissue Microsamples

Jonathan E. Mangum, Jew C. Kon, and Michael J. Hubbard

Abstract

Improved understanding of dental enamel development will benefit not only dentistry but also biomedicine more generally. Rat and mouse models of enamel development are relatively well characterized and experimentally powerful. However, the diminutive size of murine teeth makes them difficult to study using standard proteomic approaches. Here we describe gel-based proteomic methods that enable parallel quantification, identification, and functional characterization of proteins from developing rat and mouse teeth. These refined methods are also likely to be applicable to other scarce samples.

Key words: Microsample proteomics, dental development, rat and mouse models, ameloblast, sample preparation, gel electrophoresis, functional proteomics.

1. Introduction

Improved understanding of dental enamel development (amelogenesis) will not only stimulate advances in dental health but also benefit biomedical research more generally. Dentally, elucidating the causes of enamel malformations should help with their prevention in many cases, thereby saving major costs at individual and societal levels. Biomedically, better appreciation of enamel development will benefit allied topics including the cellular mechanisms of handling calcium in bulk, of avoiding calcium cytotoxicity, and of biomineralization. In pursuit of these widespread benefits, we have established proteomic approaches to query molecular and cellular aspects of amelogenesis in animal models.

We have used gel-based proteomic strategies to investigate amelogenesis in developing teeth from rats and mice. Murine teeth provide a well-characterized and powerful model of dental

development, particularly given their accessibility to genetic and pharmacological manipulations. Gel-based proteomics enables hundreds of proteins to be quantified, identified, and functionally characterized in parallel. However, the standard approaches were not well-suited to analyzing small amounts of tissue, as found in developing molars from rats and mice. This sample limitation prompted us to tailor procedures for proteomic analysis of dental tissue microsamples.

In this chapter, we describe refined methods for microsample proteomics and their application to the murine enamel epithelium. Specifically, the preparation of proteins from enamel epithelia using a serial extraction approach, customized mini-gel two-dimensional electrophoresis (2DGE), and various downstream modes of proteome analysis are outlined. These approaches have helped us elucidate mechanisms of transcellular calcium transport (1–4), functions of cytosolic calcium-binding proteins (5–10), and characteristics of a new type of molecular chaperone that we discovered in rat enamel epithelium (11–15). Many of the proteomics data generated with these methods are available on ToothPrint, a freely available online database (<http://toothprint.mdhs.unimelb.edu.au>) (16, 17). In addition to being useful for murine enamel epithelium, these methods have also proven adaptable to other scarce samples (18), suggesting a broader utility.

2. Materials

2.1. Microdissection of Enamel Epithelium and Enamel Matrix

1. Dissection buffer (*see Note 1*): 10 mM HEPES pH 7.4, 129 mM NaCl, 5 mM NaHCO₃, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1 mM CaCl₂, 1.2 mM MgSO₄, 2.8 mM glucose, store at –20°C in 50 mL aliquots.

2.2. Sequential Protein Extraction

1. TBS extraction buffer: 10 mM Tris–HCl pH 7.2, 120 mM NaCl, 10 mM ethylene glycol-bis(β-aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), plus the following protease inhibitors added just before use (*see Note 2*): 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL leupeptin, 5 μg/mL pepstatin.
2. SDS denaturant: 2% sodium dodecyl sulfate (SDS), 10 mM Tris pH 7.2, 2 mM DTT, 10 mM EGTA, plus protease inhibitors as for TBS above.
3. Benzonase (working stock 10 units/μL): made in storage buffer consisting of 50% glycerol, 20 mM Tris–HCl pH 8, 2 mM MgCl₂, 20 mM NaCl.

4. SDS/DTT: 10% SDS, 10 mM DTT.
5. Isoelectric focusing solubilization buffer (IEF-SoB): 9 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 50 mM DTT, 5% carrier ampholytes pH 3.5–10.

2.3. Gel Preparation

2.3.1. First-Dimension Carrier Ampholyte Gels

1. Glass tubes: inner diameter 1.5 mm, outer diameter 3 mm, length 7.5 cm (Sigma). Wash glass tubes by soaking overnight in 20% HCl, sonicate for 10 min in a water bath, then rinse with water until pH neutralizes. Air-dry tubes and store in a dust-free environment.
2. Acrylamide solution (*see Note 3* for safety information): pre-made 40% acrylamide solution with 2.6% cross-linker.
3. NP40/CHAPS: Nonidet P40 (NP40) and CHAPS are combined as a 10%/0.49 M solution ready for 1:18 dilution. Care should be taken when dispensing NP40, which clings to the surface of pipette tips due to its viscosity.
4. Carrier ampholytes: pH 3–10 (GE Healthcare), 3–6.5 (BDH), pH 3–5 and 4–6 (BioRad).
5. APS: ammonium persulfate made as a fresh 10% solution in water just before use.
6. Tube-gel solution: 9.25 M urea, 5% acrylamide, 1.1 mM EGTA, 0.56% NP40, 27 mM CHAPS, 2.2% carrier ampholytes (*see Table 19.1*), 0.22% APS. To dissolve urea this solution should be vortexed vigorously and sonicated in <10-s bursts (to avoid heating the solution, *see Note 4*). Just before pouring the gel, *N,N,N',N'*-tetramethyl-ethane-1,2-diamine (TEMED) is added to 0.22%, the solution is mixed and used immediately.

Table 19.1
Carrier ampholyte mixtures used for making IEF tube gels with various resolving ranges

Ampholyte premixes (pH range)	Ratio	Useful resolving range (pH)
3–6.5 and 3–10	9: 1	3.5–4.5
3–6.5 and 3–10	1: 1	4–6
3–5 and 4–6 and 3–10	1: 1: 2	4–7
3–6.5 and 3–10	1: 3	5.5–7

2.3.2.

Second-Dimension

SDS-PAGE

1. Customized gel casting plates (*see Note 5* and **Fig. 19.1**).
2. Resolving gel mixture: 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS, 0.1% TEMED, acrylamide. Note that acrylamide concentration will depend on mass range of

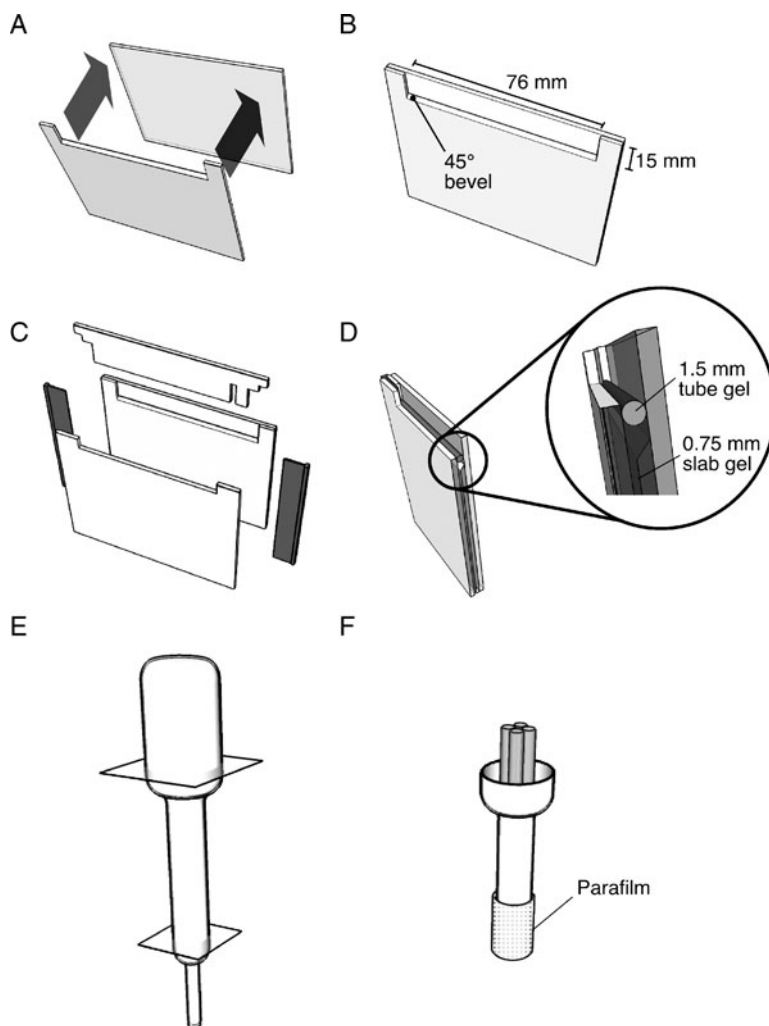


Fig. 19.1. Customized “thick/thin” gel apparatus. Customization for the Hoefer Mighty Small II system (distributed by GE Healthcare) is illustrated, but the principles should apply to any mini-gel setup. **A** Assembly of customized glass plate, using a standard Hoefer glass sheet, and a modified 0.75-mm-thick glass sheet with notch cut as specified in **B**. The two pieces are bonded with epoxy cement to produce a single unit as shown in **B**. **C** The modified glass plate is assembled with a Hoefer alumina backing plate, 0.75 mm T-spacers, and a 1.5-mm-thick preparative gel comb. **D** Cross section showing relationship between the “thick” 1.5 mm tube gel and the “thin” 0.75 mm slab gel. The role of the customized glass plate in channelling proteins from the thick tube gel into the thinner slab gel is highlighted in the magnified view. **E** Plastic transfer pipette used for casting tube gels, showing positions to be cut. **F** Assembly of gel casting apparatus with glass tubes protruding ~ 0.7 cm above the *top* and Parafilm sealing the base.

interest. We routinely use 12.5% acrylamide for resolving 15–250 kDa proteins.

3. Gel combs: 1.5 mm preparative gel combs (BioRad), create one narrow lane for mass ladder and one wide lane for tube gel.

2.4. Gel Electrophoresis

2.4.1. First-Dimension Carrier Ampholyte Gels

1. Catholyte (upper) running solution: 20 mM sodium hydroxide, diluted from 0.2 M stock that is stored at 4°C and replaced every 4 weeks. Just before IEF, the catholyte running solution is degassed by bath sonication for 5 min at 30°C (*see Note 6*).
2. Anolyte (lower) running solution: 0.03% (v/v) phosphoric acid, diluted from 85% aqueous solution (AR grade) just before use.

2.4.2. Second-Dimension SDS-PAGE

1. Laemmli running buffer: 25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS.
2. Transfer buffer: 70 mM Tris-HCl pH 6.8, 3% SDS, 0.002% bromophenol blue.

3. Methods

Developing murine molars provide limited amounts of tissue (e.g., two mandibular first molars from a 5-day-old rat yield ~6 μ L enamel epithelium, and a mouse gives half this amount). Such limitations pose a challenge for standard immobilized pH gradient (IPG) 2DGE. To maximize experimental yields and reduce animal usage, we developed a higher sensitivity approach based on mini-format, carrier ampholyte-based 2DGE in combination with sequential protein extraction. Carrier ampholyte isoelectric focusing (CA-IEF) provides superior protein-loading capacity and recovery over IPG-based IEF (17). Customized mini-gels are used to interface “thick” CA-IEF tube gels with “thin” slab gels for the second dimension, increasing sensitivity by concentrating individual proteins into smaller gel volumes. Sequential protein extraction reduces sample complexity by producing, in our case, three fractions that each contain distinct protein populations associated with cellular components (i.e., cytosol, membranes, or nucleus/cytoskeleton; *see Fig. 19.2*). This fractionation boosts detection sensitivity, provides links to subcellular location, and effectively compensates for the limited spatial resolution of

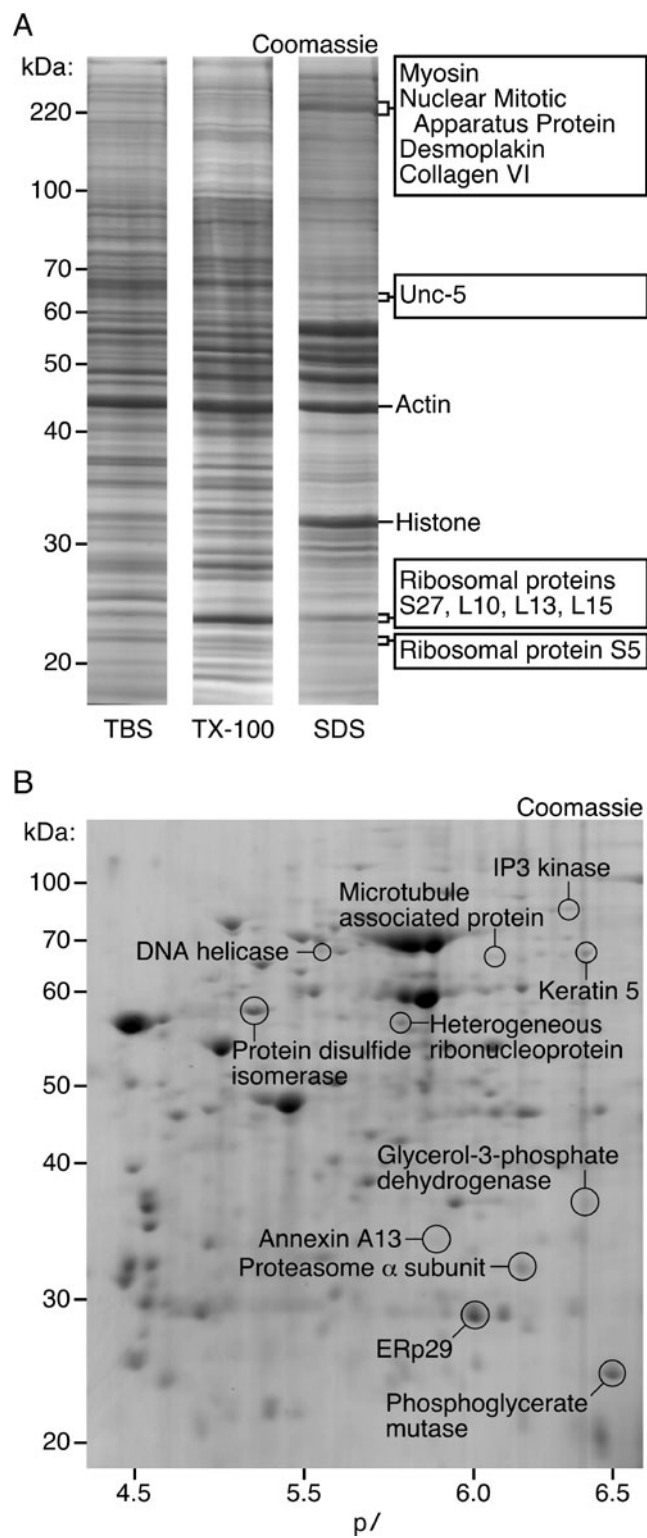


Fig. 19.2. Gel-based identification of enamel cell proteins. **A** SDS-PAGE analysis of fractions obtained from rat enamel epithelium following sequential extractions with

mini-gels. Using these methods we have resolved ~500 Coomassie-stained gel spots using enamel epithelium from a single rat neonate (19). In combination with blot-based analyses, 2DGE can be married with a variety of downstream procedures that enable the quantitation, identification, and functional characterization of many proteins in parallel.

3.1. Microdissection of Enamel Epithelium and Enamel Matrix

1. Mandibular first molars are isolated during the secretion or maturation phase of amelogenesis (i.e., from 4–5 and 9–10-day-old pups, respectively) (6, 20). Enamel epithelium is subsequently microdissected under ice-cold dissection buffer as rapidly as practicable (generally completed within 5–7 min after killing the pup) to minimize post-mortem modification of proteins (e.g., proteolysis). Epithelia from each animal are transferred to a fresh 1.5 mL centrifuge tube (*see* **Note 7**), excess liquid is removed using a gel-loading pipette tip, and then the tissue is frozen over dry ice and stored at -80°C .
2. Enamel proteins are isolated from the extracellular matrices that remain after epithelial isolation (Step 1) (1). Gently scrape the exposed enamel matrix surface with a micro-knife to release the soft layer of partially mineralized enamel proteins from underlying dentine (which is firmer and distinctly colored). This white particulate material is then pooled and sedimented by centrifugation (1,000*g* for 2 min). The enamel matrix pellet is dissolved in 10 volumes of 4% trifluoroacetic acid, assisted by bath sonication. Next, SDS and EGTA are added (1% and 100 mM, respectively) along with a trace of bromophenol blue, then the mixture is dried in a vacuum centrifuge. The dried pellet is dissolved in the original volume of water and the pH neutralized with ammonia vapor as required (hold a small droplet at the end of a pipette tip close to the sample surface until the solution turns from yellow to blue). Next, Tris-HCl pH 7.2 and dithiothreitol are added to final concentrations of 25 mM and 10 mM,

Fig. 19.2. (continued) Tris-buffered saline (TBS), Triton X-100, and SDS as indicated. Distinct banding patterns are evident after Coomassie staining, consistent with sampling of different cellular compartments as intended (nominally cytosol, organelles, and cytoskeleton/nucleus, respectively). Four bands from the SDS-soluble lane subjected to gel-LC/MS² analysis and the proteins identified therein are boxed. Actin and histone bands, identified previously, are indicated. **B** 2DGE analysis of the TBS-soluble fraction from **A**, illustrating the effective isolation of numerous proteins. Only the acidic region of this Coomassie-stained gel is shown. Protein identifications made to establish the limit of sensitivity are indicated, and other abundant proteins have been reported elsewhere (8, 17). This figure was taken from (19) and reproduced with permission from Blackwell Publishing.

respectively. EGTA is added incrementally (1 μL additions from a 1 M, pH 7 stock) until any remaining calcium dodecyl sulfate (visible as a white precipitate) is dissolved. Finally, boil the sample for 5 min, centrifuge (17,000*g* for 2 min), and store at -80°C .

3.2. Sequential Protein Extraction

1. Two frozen enamel epithelia are thawed in 30 μL of ice-cold TBS extraction buffer, then vortexed and bath sonicated for 5 min to homogenize the tissue. Homogenates are then frozen over dry ice for 5 min. After thawing on ice, this vortex/sonicate/freeze cycle is repeated three times (*see Note 8*). After the fourth cycle is completed, the homogenate is centrifuged at 22,000*g* for 5 min (4°C). The supernatant (nominal *cytosolic fraction*, *see Note 9*) is collected. The pellet is washed with 30 μL of TBS extraction buffer, centrifuged again, and the ensuing supernatant is pooled with the cytosolic fraction.
2. The pellet is then taken up in 30 μL of TBS extraction buffer containing 2% Triton X-100, vortexed and sonicated for 1 min, then incubated for 15 min at 4°C with intermittent vortexing/sonication. The extract is centrifuged as above and the supernatant (nominal *organellar fraction*) is collected. Wash the pellet with 30 μL TBS extraction buffer containing 2% Triton X-100, centrifuge again, and pool the supernatant as above.
3. The pellet is then taken up in 30 μL of SDS denaturant, vortexed and sonicated for 1 min, then boiled for 2 min. The extract is centrifuged at 22,000*g* for 5 min at 20°C , and then the supernatant (nominal *cytoskeletal/nuclear fraction*) is collected and stored on ice. The pellet is washed with 30 μL of SDS denaturant, centrifuged again, and the supernatant is pooled as above.
4. Before the extracts are used for 2DGE or SDS-PAGE (*see Note 10*), they should be treated to degrade any contaminating nucleic acid and subsequently ethanol precipitated to remove salts/small molecules and concentrate the sample.
5. To degrade nucleic acids, 1 μL (10 units) of Benzonase is added (*see Note 11*) per 60- μL extract and incubated on ice for 30 min (*see Note 12*). To assist protein solubilization after ethanol precipitation, 3 μL of SDS/DTT is added and the extracts are incubated at 100°C for 2 min. After cooling on ice for 1 min, 9 volumes of cold (-20°C) ethanol is added. Samples are incubated at -20°C for at least 30 min to maximize protein precipitation and then centrifuged at 14,000*g* for 5 min (4°C). The supernatant is discarded and residual ethanol completely removed under a gentle stream

of dry nitrogen or with a vacuum centrifuge. Next, 3 μL of SDS/DTT is added directly onto the dried pellet and the tube is vortexed for 1 min before adding 20 μL of IEF-SoB (*see* **Notes 13 and 14**). The pellet is completely dissolved by repeated pipetting (avoid creating air bubbles), and then the samples are centrifuged (20,000*g* for 5 min at 25°C) to sediment particulates that might occlude the loading surface of the tube gel. Samples can be stored at -80°C before 2DGE, but if so should be thoroughly mixed and centrifuged again before loading.

3.3. Gel Preparation

3.3.1. First-Dimension Carrier Ampholyte Gels

1. A disposable 3-mL plastic transfer pipette is adapted to produce a tube-gel casting chamber. Four glass tubes are placed inside the chamber and Parafilm is used to seal the base (*see* **Fig. 19.1** and **Note 15**). Tube gels should be positioned as close to vertical as possible (e.g., place casting chamber inside a 15-mL Falcon tube which is standing upright in a storage rack).
2. Note: rapidly complete this step (<2 min) to ensure even polymerization of acrylamide. The tube-gel solution is pipetted into the casting chamber between the gaps of adjacent tubes, making ~ 0.2 -mL additions and moving around the perimeter of the chamber with each addition. Water containing 0.002% bromophenol blue (overlay solution) is added in the same manner to displace the tube-gel solution up into the glass tubes. Addition of the blue overlay solution is continued until the colorless tube-gel solution is ~ 7 mm from the top of the glass tubes (*see* **Note 16**). To ensure a flat surface for sample loading and to prevent dehydration of gels during polymerization, 5 μL of overlay solution is gently added on top of the acrylamide in each glass tube using a gel-loading tip. If sample volumes greater than 20 μL are to be loaded, the amount of overlay solution can be increased as appropriate.
3. After initial polymerization has proceeded for 15 min at room temperature, the top of the casting tube is gently covered with stretched Parafilm to prevent dust contamination and reduce evaporation of overlay buffer. Complete polymerization of acrylamide minimally requires incubation for a further 45 min at room temperature.
4. Once the tube gels have completely polymerized, gently peel the Parafilm from both ends of the casting chamber and eject the glass tubes. The lower polyacrylamide “plug” is carefully excised from the tubes with a clean razor blade. It is important at this step that the base of the tube gel

is not stretched or otherwise disturbed, because resulting discontinuities between the gel and glass tube interior (i.e., “bubbles”) are a common cause of current leak during IEF. Remove residual polyacrylamide from the glass tube exterior with a lint-free tissue. Remove liquid from the top of the gel using a gel-loading tip while taking care to avoid disturbing the loading surface (*see* **Notes 17 and 18**). Once all tubes are clean and checked for integrity (reject any tubes with bubbles) their ends are sealed with Parafilm, again taking particular care with the base of the tubes. Finally, the tubes are wrapped in cling-film, labeled (date, ampholyte mix), and then stored at 4°C for up to 2 weeks.

3.3.2.

Second-Dimension SDS-PAGE

1. Gel plates are assembled inside the caster with 0.75-mm spacers according to the manufacturer’s instructions.
2. Resolving gel mixture is poured into the caster until the gel cassettes are full, and then 1.5-mm-thick preparative combs are inserted (*see* **Note 19**).
3. Gels are incubated at room temperature for at least 1 h before the caster is disassembled. The gels are cleaned then wrapped in cling-film together with a moistened paper towel (to maintain gel hydration) and stored at 4°C for up to 2 weeks.

3.4. Gel

Electrophoresis

3.4.1. First-Dimension Carrier Ampholyte Gels

1. Tubes are assembled into a focusing rack according to the manufacturer’s instructions (care should be taken to align the tube tops as closely as possible). Up to 12 tube gels can be focused concurrently without having to change the standard power settings (*see* below). Samples in IEF-SoB are loaded into each tube using a gel-loading pipette tip and, if the tube is not full after loading, it is topped up with IEF-SoB. Once all samples have been loaded, the rack is mounted onto the gel-running rig. The catholyte running solution is poured slowly into the upper chamber until all tubes are just submerged (by 1–2 mm). The anolyte running solution is poured into the lower chamber, fully submerging the lower ends of the tubes.
2. Isoelectric focusing is initially performed with power settings of 200 V, 5 mA, 1.5 W, and without cooling. Once the current has reduced to ~0.8 mA (typically this takes 30–45 min), the power settings are changed to 1,000 V, 2.5 mA, 1.5 W, and water cooling of the gel rig is started. Normally it takes 30–60 min to reach 1,000 V, depending on the number of tubes being focused (more tubes

take longer). Progress of this IEF step is tracked by recording the time, voltage, current, watts, and cumulative volt-hours (Vh) every 15–30 min. If the voltage fails to reach 1,000 V after 2 h of focusing there is likely to be a current leak, which requires intervention (*see* **Note 20**). For tissue extracts, IEF is typically optimal once a total of 3,200–3,600 Vh has been reached (note that somewhat shorter focusing times may be beneficial for less complex samples). The focusing rack is removed and immediately placed on ice to reduce defocusing (diffusion) of proteins while awaiting extrusion.

3. To eject each gel, the glass tube is connected to a water-filled syringe using an extrusion adapter (available from Sigma). The basic (lower) end of the tube is placed on a piece of Parafilm and then the syringe plunger is pressed gently until the gel begins to emerge. Once ~1 cm of gel has emerged, a yellow pipette tip is placed on it to prevent flipping should the subsequent extrusion be poorly controlled (e.g., due to over-pressurization). Once the gel is fully extruded, excess water is removed with a lint-free tissue and the Parafilm is labeled to indicate gel orientation and sample identity. The Parafilm-supported gel is then placed inside a 15-mL screw-cap Falcon tube and snap-frozen on dry ice. Tube gels are stored at –80°C until ready for second-dimensional separation. Used glass tubes are stored submerged in water before cleaning as described (**Section 2.3.1**).

3.4.2. Second-Dimension SDS-PAGE

1. Resolving gels are assembled into the gel-running rig and Laemmli running buffer is added to the upper chamber until it almost reaches the level of the loading well. The comb is carefully removed and any liquid remaining in the loading well is removed with a gel-loader tip (this helps prevent the tube gel from floating when running buffer is added in Step 3, below).
2. Tube gels (on Parafilm) are thawed by adding 200 µL of transfer buffer and incubated for 2 min at room temperature (*see* **Notes 21 and 22**). When completely thawed the gel appears optically clear, with no sign of bubbles.
3. Transfer buffer is drained and the tube gel is loaded by slowly lowering one end into the loading well with the help of a thin metal spatula. The rest of the tube gel is gradually placed into the well, working from one end to the other, taking care to avoid trapping air bubbles against the resolving gel (*see* **Note 23**). Any trapped bubbles should be removed carefully by tapping gently on the tube gel with a spatula. Running buffer is then carefully topped up to immerse the tube gel and any further bubbles are removed as before.

Molecular weight markers are also loaded if desired (*see* **Note 24**). Second-dimension separation is performed at 200 V and 12–15 mA per gel and continued until the dye front reaches the bottom of the resolving gel.

3.5. Protein Analysis

3.5.1. Protein Quantitation

To quantify proteins we routinely use densitometric analysis after staining gels with Coomassie Brilliant Blue, which provides a good dynamic range (unlike silver staining) and interfaces well with protein identification technologies including mass spectrometry (**Fig. 19.2** and (19)). Although higher detection sensitivities can be achieved with fluorescent stains, these methods might compromise mass spectrometry (21). Quantitative immunoblotting is a powerful adjunct approach for proteins of particular interest, when suitable antibodies are available (22).

3.5.2. Protein Identification

For identification by mass spectrometry of in-gel tryptic digests, proteins stained with Coomassie Blue are preferred for reasons of sensitivity and sequence coverage. With 2DGE protein spots, MALDI-TOF-based peptide mass fingerprinting is generally sufficient for identification when combined with the electrophoretic properties (M_r and pI). For SDS-PAGE bands, LC-MS/MS sequence tags ($n \geq 2$) can provide identification of several proteins from a single band, albeit with reduced quantitative information (**Fig. 19.2** and (19)). In all cases, however, it is imperative to substantiate functionally important identifications with additional measures (e.g., immunoblotting or functional characterization as described in the following section) (11).

3.5.3. Functional Characterization

After electrophoresis, proteins can be electroblotted onto a membrane (nitrocellulose or PVDF) and functionally probed with potential ligands. For example, protein-binding or calcium-binding functionality may be determined in this way (6, 7, 23). Immunoblotting can also be used for targeted analyses of protein heterogeneities, such as isoforms or post-translational modifications (e.g., phospho/glyco forms or amidation states) (6, 12).

4. Notes

1. All solutions are made in ultrapure water (MilliQ, 18.2 M Ω cm). All reagents are electrophoresis or analytical grade and purchased from Sigma or BDH unless stated otherwise. Buffers are diluted from 1 M stocks, the pH of which is measured at room temperature. The pH of working

solutions should not be adjusted after mixing buffer stocks with other ion-containing solutions.

2. A cocktail of protease inhibitors is used in an effort to protect against multiple classes of proteases. Benzamide (trypsin inhibitor), pepstatin (aspartic acid protease inhibitor), and leupeptin (serine/cysteine protease inhibitor) are all stable in water. However, PMSF (serine protease inhibitor) undergoes autohydrolysis in aqueous solution ($t_{1/2} < 2$ h at pH 7 (24)) and so must be stored in anhydrous organic solvent (we use a 0.1 M stock in neat ethanol). It is primarily due to the instability of PMSF that protease inhibitors are added just before homogenizing enamel epithelia.
3. Although acrylamide premixed in aqueous solution is safer to use than the powder form, protective equipment (lab coat, gloves, safety glasses) still must be employed when handling this potent neurotoxin.
4. When handling urea in aqueous solution, it is important to avoid low and high temperatures. Temperatures below 15°C will cause urea to precipitate, decreasing its protein-solubilizing capacity. Temperatures above 37°C can result in degradation of urea to reactive cyanate species that in turn can carbamylate nucleophilic amino acids (lysine, arginine, cysteine, and initiator methionine). Such carbamylation is to be avoided because it introduces charge heterogeneity to proteins, which manifests as acidic spot trains on 2DGE (*see* (25) and references therein).
5. Glass plates are modified to allow 1.5-mm diameter tube gels to be interfaced with 0.75-mm-thick resolving gels. This is beneficial because thin resolving gels provide higher protein concentration in each gel spot, thereby improving detection sensitivity and the reliability of protein identification from in-gel digests.
6. Thoroughly degassing the catholyte buffer just prior to use is widely thought to be important for achieving good IEF results. Failure to do this properly can lead to gas bubble formation inside the tube (above the gel surface) as the apparatus warms, resulting in current disruption and underfocusing.
7. All procedures use low protein-binding microcentrifuge tubes (e.g., Eppendorf LoBind). Such tubes minimize the amount of protein that is lost to the tube wall, obviating the need for surface pre-treatments such as siliconization. Besides providing good protein recovery, these tubes have a low content of plasticizer, which is important for downstream mass spectrometry.

8. We have found this freeze/thaw homogenization approach gives greater protein recovery than traditional micro-pestle homogenization approaches, presumably due to reduced sample handling and loss to surfaces.
9. The freeze–thaw procedure produces a soluble fraction (nominally cytosol) due to rupture of the plasma membrane by volume expansion during ice formation. Insoluble complexes of cytosolic proteins (e.g., cytoskeleton) and unbroken organelles (e.g., ER, mitochondria, nuclei) remain in the pellet predominantly. Subsequent Triton solubilization of the pellet produces an organelle-enriched fraction by releasing membranous and luminal proteins, while insoluble cytoskeletal proteins and DNA-bound proteins remain in the pellet. SDS solubilizes many of the latter, and subsequent DNase treatment releases any remaining DNA-bound proteins.
10. All three extracts can be applied to 2DGE; however, it should be kept in mind that some proteins in the Triton and SDS fractions may not be amenable to IEF. For example, when several proteins from the SDS fraction were analyzed by SDS-PAGE and LC-MS (gel-LC-MS), we found the majority were too hydrophobic, too large, or too highly charged to be visualized by 2DGE (19). Hence a combination of 2DGE and gel-LC-MS can be used to maximize coverage of the enamel epithelium proteome.
11. Benzonase is a DNA/RNA nuclease which is particularly useful due to its resilience against harsh conditions (e.g., 7 M urea, 1% SDS, pH 6–10, 0–40°C) (26).
12. Two epithelia from a rat neonate provide sufficient protein for Coomassie-stained 2DGE, as determined empirically (19). SDS-PAGE requires approximately 30% this amount, reflecting the broader range of proteins resolved by the gel.
13. SDS/DTT is added to pellets to maximize protein solubility in IEF-SoB and so reduce subsequent protein precipitation during initial stages of IEF. The amount of SDS/DTT was determined empirically to improve protein solubility and yet not impair resolution.
14. Carrier ampholyte IEF gels are able to accommodate substantial amounts of SDS/DTT due to the open nature of the system. Protein is initially electrophoresed into the tube gel before the carrier ampholytes migrate to their pI positions. A current is generated during this initial electrophoresis phase, which is sufficient to facilitate rapid migration of charged SDS, DTT, and other small ions out of the tube gel into the running solution.

15. A larger number of tube gels (~25) can be cast by using a modified 15 mL disposable syringe. The needle outlet is removed to create an open-ended cylinder, and the plunger is used to seal the base and later provide a gentle means for ejecting the cast tubes.
16. It is important to empirically determine the volume of overlay buffer needed to cast tube gels of a desired length. A substantial plug of polyacrylamide gel is required at the bottom of the caster (≥ 2 cm from base) to maintain the intended acrylamide concentration inside the tubes.
17. It is important to remove the overlay solution to prevent dilution of urea at the gel surface during storage. A high concentration of urea is needed to maximize sample entry to the gel.
18. After polymerization, acrylamide properties can be assessed using a gel-loading pipette tip; properly prepared gels feel quite firm. If the gel is able to be drawn into the pipette tip and appears viscous, the polyacrylamide concentration at the loading surface is too low – the batch of tube gels should be rejected.
19. We routinely omit stacking gels for simplicity and reproducibility, as suggested by others (27).
20. Maximum voltage is considered to be more important for spot resolution than length of focusing time, so ensure that 1,000 V is reached (by minimizing current leaks).
21. Generally we do not reduce and alkylate tube gels before second-dimension electrophoresis because samples are heavily reduced before loading and our transfer procedure is rapid enough to avoid cysteine oxidation. By avoiding this step, sensitivity and resolution are improved through both reducing sample loss from the gel (i.e., washout during transfer) and minimizing protein diffusion in the tube gel.
22. Time spent equilibrating the tube gel in transfer buffer can be critical. Too long and proteins can be lost, too short and the CHAPS/NP40 in the tube gel may not be displaced by SDS (resulting in loss of small acidic proteins, particularly).
23. It is important to completely thaw the gels before loading. Incompletely thawed gels produce minute gas bubbles, which can cause vertical streaking of 2DGE maps.
24. Regular mass standards (in solution) will give approximate mass (M_r) comparisons only. For accurate mass calibration the M_r markers need to be cast inside a tube gel, which is then cut to size and placed into the marker lane.

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References

1. Hubbard, M. J. (1996) Abundant calcium homeostasis machinery in rat dental enamel cells. Up-regulation of calcium store proteins during enamel mineralization implicates the endoplasmic reticulum in calcium transcytosis. *Eur. J. Biochem.* **239**, 611–623.
2. Hubbard, M. J. (2000) Calcium transport across the dental enamel epithelium. *Crit. Rev. Oral Biol. Med.* **11**, 437–466.
3. Franklin, I. K., Winz, R. A., and Hubbard, M. J. (2001) Endoplasmic reticulum Ca^{2+} -ATPase pump is up-regulated in calcium-transporting dental enamel cells: a non-housekeeping role for SERCA2b. *Biochem. J.* **358**, 217–224.
4. Turnbull, C. I., Looi, K., Mangum, J. E., Meyer, M., Sayer, R. J., and Hubbard, M. J. (2004) Calbindin independence of calcium transport in developing teeth contradicts the calcium ferry dogma. *J. Biol. Chem.* **279**, 55850–55854.
5. Hubbard, M. J., and McHugh, N. J. (1995) Calbindin28kDa and calbindin30kDa (calretinin) are substantially localised in the particulate fraction of rat brain. *FEBS Lett.* **374**, 333–337.
6. Hubbard, M. J. (1995) Calbindin28kDa and calmodulin are hyperabundant in rat dental enamel cells. Identification of the protein phosphatase calcineurin as a principal calmodulin target and of a secretion-related role for calbindin28kDa. *Eur. J. Biochem.* **230**, 68–79.
7. Hubbard, M. J., and McHugh, N. J. (1996) Mitochondrial ATP synthase F1-beta-subunit is a calcium-binding protein. *FEBS Lett.* **391**, 323–329.
8. Hubbard, M. J. (1998) Enamel cell biology. Towards a comprehensive biochemical understanding. *Connect. Tissue Res.* **38**, 17–32.
9. Hubbard, M. J. (1998) Proteomic analysis of enamel cells from developing rat teeth: big returns from a small tissue. *Electrophoresis* **19**, 1891–1900.
10. Sayer, R. J., Turnbull, C. I., and Hubbard, M. J. (2000) Calbindin28kDa is specifically associated with extranuclear constituents of the dense particulate fraction. *Cell Tissue Res.* **302**, 171–180.
11. Demmer, J., Zhou, C., and Hubbard, M. J. (1997) Molecular cloning of ERp29, a novel and widely expressed resident of the endoplasmic reticulum. *FEBS Lett.* **402**, 145–150.
12. Hubbard, M. J., and McHugh, N. J. (2000) Human ERp29: isolation, primary structural characterisation and two-dimensional gel mapping. *Electrophoresis* **21**, 3785–3796.
13. Hubbard, M. J., McHugh, N. J., and Carne, D. L. (2000) Isolation of ERp29, a novel endoplasmic reticulum protein, from rat enamel cells: Evidence for a unique role in secretory-protein synthesis. *Eur. J. Biochem.* **267**, 1945–1957.
14. Hubbard, M. J., Mangum, J. E., and McHugh, N. J. (2004) Purification and biochemical characterisation of native ERp29 from rat liver. *Biochem. J.* **383**, 589–598.
15. Hermann, V. M., Cutfield, J. F., and Hubbard, M. J. (2005) Biophysical characterization of ERp29. Evidence for a key structural role of cysteine 125. *J. Biol. Chem.* **280**, 13529–13537.
16. Hubbard, M. J., Faught, M. J., Carlisle, B. H., and Stockwell, P. A. (2001) ToothPrint, a proteomic database for dental tissues. *Proteomics* **1**, 132–135.
17. Hubbard, M. J., and Kon, J. C. (2002) Proteomic analysis of dental tissues. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **771**, 211–220.
18. Mangum, J. E., Farlie, P. G., and Hubbard, M. J. (2005) Proteomic profiling of facial development in chick embryos. *Proteomics* **5**, 2542–2550.
19. Mangum, J. E., Veith, P. D., Reynolds, E. C., and Hubbard, M. J. (2006) Towards second-generation proteome analysis of murine

- enamel-forming cells. *Eur. J. Oral Sci.* **114**(Suppl. 1), 259–265.
20. Kardos, T. B., and Hubbard, M. J. (1981) Rapid dissection of rodent molar-tooth germs. *Lab. Anim.* **15**, 371–373.
 21. Lanne, B., and Panfilov, O. (2005) Protein staining influences the quality of mass spectra obtained by peptide mass fingerprinting after separation on 2-d gels. A comparison of staining with coomassie brilliant blue and sypro ruby. *J. Proteome Res.* **4**, 175–179.
 22. Shnyder, S. D., Mangum, J. E., and Hubbard, M. J. (2008) Triplex profiling of functionally distinct chaperones (ERp29/PDI/BiP) reveals marked heterogeneity of the endoplasmic reticulum proteome in cancer. *J. Proteome Res.* **7**, 3364–3372.
 23. Hubbard, M. J., and Klee, C. B. (1987) Calmodulin binding by calcineurin. Ligand-induced renaturation of protein immobilized on nitrocellulose. *J. Biol. Chem.* **262**, 15062–15070.
 24. James, G. T. (1978) Inactivation of the protease inhibitor phenylmethylsulfonyl fluoride in buffers. *Anal. Biochem.* **86**, 574–579.
 25. McCarthy, J., Hopwood, F., Oxley, D., Laver, M., Castagna, A., Righetti, P. G., Williams, K., and Herbert, B. (2003) Carbamylation of proteins in 2-D electrophoresis – myth or reality? *J. Proteome Res.* **2**, 239–242.
 26. Biedermann, K., Jepsen, P. K., Riise, E., and Svendsen, I. (1989) Purification and characterization of a *Serratia marcescens* nuclease produced by *Escherichia coli*. *Carlsberg Res. Commun.* **54**, 17–27.
 27. Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A. C., Miller, M. J., and Merrill, C. R. (1988) Methods for increasing the resolution of two-dimensional protein electrophoresis. *Anal. Biochem.* **173**, 424–435.

Chapter 20

Immunological Techniques: ELISA, Flow Cytometry, and Immunohistochemistry

Pauline J. Ford

Abstract

Techniques to analyze the host immune response elicited by the presence of oral microorganisms and their products are central to our understanding of the local and systemic effects of oral diseases. This immune response has been extensively investigated for periodontal disease. The local response may result in lesions involving the gingival tissues and depending upon host susceptibility and microbial virulence may lead to local tissue destruction. More recently, however, the importance of the systemic inflammatory and immune response to oral organisms has been recognized. These systemic responses have been associated with an increased risk for cardiovascular disease, diabetes, and preterm low birth weight. A number of techniques are used extensively by researchers investigating humoral and cellular immune responses to oral organisms both in local oral tissues and fluids and systemically in peripheral blood. These are enzyme-linked immunosorbent assay (ELISA) to quantify specific antibody and cytokines in serum, gingival crevicular fluid (GCF), and saliva; characterization of T cells from peripheral blood and gingival tissues using flow cytometry; and immunohistological analysis of the inflammatory cell infiltrate in gingival tissues.

Key words: ELISA, antibody, serum, gingival crevicular fluid, saliva, T cells, flow cytometry, immunohistology.

1. Introduction

The oral microbiota consists of a complex, interacting community of microorganisms which is acquired soon after birth and adapts to the changing oral environment over time. While this microbiota is essential for the maintenance of oral health, microbial adaptation to altered oral conditions may result in infectious oral disease (dental caries, periodontal disease, candidiasis) (1).

The immune response of the host to the organisms implicated in the pathogenesis of oral disease has been studied in some detail

over the last few decades. Determination of levels of cytokines and of specific antibody to putative oral pathogens by ELISA has been a fundamental part of these studies. Fluids analyzed are serum and less commonly gingival crevicular fluid (GCF) and saliva (2–4). Although the composition of GCF and saliva may be altered by local tissue responses, they are primarily serum derivatives, so care must be taken with interpretation of results. Techniques to analyze the local and systemic T-cell response are more labor and resource intensive, however yield valuable data on cellular responses (5–8). These techniques include cell culture and profiling using flow cytometry. Detection and localization of specific cellular markers by immunohistology provides useful spatial information on events occurring at the local tissue level and can also be analyzed quantitatively (6, 9, 10). The range of commercially available antibodies is extensive, allowing identification of virtually any known cellular antigen. Samples however are more problematic to obtain than biological fluid samples since gingival biopsy is required.

There has been increasing evidence for the role of oral inflammation and infection in the pathogenesis of systemic diseases, particularly cardiovascular disease, diabetes, and preterm low birth weight (11). The techniques described here are useful tools for analyzing the associations of oral disease with systemic inflammation, systemic immune responses to oral organisms, and systemic disease.

2. Materials

2.1. Sample Collection and Processing

2.1.1. Peripheral Blood

1. BD Vacutainer[®] potassium EDTA tubes for serum collection or sodium heparin tubes for mononuclear cell collection (*see Note 1*).
2. Flashback blood collection needle (21 gauge).
3. Multiple sample sleeve and tourniquet.
4. Collection of mononuclear cells: Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden), RPMI-1640 (Sigma, St Louis, Mo), 50-mL Falcon tubes (BD Biosciences, San Jose, CA), 20-mL syringes, mixing cannulae.

2.1.2. GCF

1. Filter strips (Millipore[®]).
2. Sterile periodontal curette.

3. Sterile 0.5-mL plastic tubes containing 10 mM NaH₂PO₄ and 150 mM NaCl, pH 7.2.

2.1.3. Tissues

1. Sterile specimen containers.
2. Transport medium: RPMI-1640 (Sigma) containing 10% fetal calf serum (Gibco, Paisley, Scotland, UK), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 1.25 µg/mL amphotericin B (*see Note 2*).
3. Histology requirements:
 - (a) Thirty percentage sucrose in PBS.
 - (b) Tissue-Tek[®] OCT (Sakura Finetek USA, Inc., Torrance, CA).
 - (c) Disposable moulds (Canemco, Inc. and Marivac, Inc., Quebec, Canada) (*see Note 3*).
 - (d) Liquid nitrogen.
 - (e) Glass slides.
 - (f) Cryostat.
 - (g) Fixing solution: Equal parts of acetone and chloroform.
4. T-cell culture requirements:
 - (a) Scalpel.
 - (b) Digestion medium: RPMI-1640 (Sigma) containing 10% heat-inactivated pooled human AB serum, 1% glutamine (Sigma), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 1.25 µg/mL amphotericin B.
 - (c) 1 mg/mL Collagenase type 2 (Sigma).
 - (d) Tube roller.
 - (e) Cell strainers (BD Biosciences).
 - (f) RPMI-1640.
 - (g) Ficoll-Paque (Pharmacia LKB).
 - (h) Fetal calf serum (Gibco).
 - (i) 15-mL Falcon tubes (BD Biosciences).
 - (j) 20-mL Syringes.
 - (k) Mixing cannulae.
 - (l) Culture medium: RPMI-1640 (Sigma) containing 10% heat-inactivated pooled human AB serum, 1% glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Sigma), and 1.25 µg/mL amphotericin B.

2.2. ELISA

The method described is for detection of IgG antibodies in human serum or GCF samples. Using an appropriate secondary antibody and standard, this method can be adapted to measure

other antibody isotypes and subclasses and also for the determination of antibody levels in serum samples from experimental animals (12, 13).

1. 96-Well high-binding plates (MaxiSorb immunoplates; Nunc, Roskilde, Denmark).
2. Adhesive plate sealers.
3. 0.05 M Carbonate buffer (pH 9.6): 1.59 g Na_2CO_3 and 2.93 g NaHCO_3 in 1 L of distilled water.
4. Phosphate buffered saline (PBS), pH 7.2.
5. PBS with 0.05% Tween 20 (PBS Tween).
6. PBS Tween with 1% bovine serum albumin (Invitrogen, Mount Waverley, Australia).
7. 2.5 mM *o*-Tolidine (Eastman Kodak, Rochester, NY).
8. 3% H_2O_2 .
9. Citrate buffer (pH 4.5): Solution A: 14.2 g Na_2HPO_4 and 0.01 g disodium EDTA in 1 L distilled water; solution B: 21.01 g citric acid in 1 L distilled water. Add solution B to solution A until pH 4.5 is reached.
10. 3 M HCl.
11. Antigen to coat plates (1 $\mu\text{g}/\text{mL}$ in carbonate buffer).
12. Sample diluted in PBS (usually in the range of 1:10–1:100 depending on the concentration of antibody in sample).
13. Secondary antibody [peroxidase-conjugated anti-human IgG (DAKO, Glostrup, Denmark) diluted 1:5,000 in PBS Tween].
14. Standard: Human IgG (Zymed, CA).
15. Plate washer (Ultrawash Plus plate washer; Dynatech Labs, Chantilly, VA).
16. Microplate reader (BIO-RAD, Model 3550).

2.3. T-Cell Culture

T-cell cultures can be established from the peripheral blood or from gingival tissue samples. Ficoll-Paque density centrifugation is required for either sample type to isolate the mononuclear cells and to remove the majority of other cell types and debris.

1. Hemocytometer chamber and coverslip.
2. Ethidium bromide in PBS (2 mg/mL) and acridine orange in PBS (2 mg/mL). Store stock solutions in amber bottles at 4°C. These stains are suspected carcinogens. Refer to material safety data sheets for details on precautions for use, storage, and disposal.
3. Fluorescence microscope with UV lamp.
4. Culture medium.

5. Flat-bottomed 24-well tissue culture plates (Nunc).
6. CO₂ incubator.
7. Gamma cell.
8. RPMI-1640 containing 10% dimethyl sulfoxide (DMSO) and 20% fetal calf serum (FCS) (add DMSO to RPMI, then sterile filter before adding FCS; 5 mL aliquots can be stored at -20°C).
9. Cryogenic controlled rate freezing container (Nalgene).
10. Antigen for stimulation of specific T cells (for example, outer membrane preparation of *Porphyromonas gingivalis*).
11. 15-mL Falcon tubes.
12. Recombinant human interleukin-2 (Boehringer Mannheim, Mannheim, Germany).

2.4. Flow Cytometry

For intracellular antigens to be detected, cells must be fixed and permeabilized to allow the antibodies to penetrate the cell membrane and the membranes of the endoplasmic reticulum and Golgi apparatus. This method stains cell surface antigens, then allows staining of the intracellular antigens while preserving the cellular morphology and antigen-binding site availability (11).

1. Flow cytometer.
2. Sheath fluid (sterile-filtered PBS).
3. 50-mL Falcon tubes.
4. Fluorescent-labeled antibodies.
5. Falcon polystyrene round-bottomed tubes 12 mm × 75 mm (BD).
6. Vacuum aspirator.
7. PBS azide: 1.3 g Sodium azide in 1 L PBS.
8. FACS fixative: 20 g Glucose and 26 mL formaldehyde in 1 L PBS azide (wrap bottle in foil and store at 4°C).
9. Paraformaldehyde PBS: 4 g Paraformaldehyde in 100 mL PBS. Add 80 mL PBS to 4 g paraformaldehyde, heat to 60°C while stirring in fume hood. Add 2–3 drops of 4 N NaOH and continue heating while stirring until dissolved. This takes about 10 min. Cool, then adjust pH to 7.2. Make up to a total volume of 100 mL.
10. Proteinase K buffer: Solution A (1 M Tris): 12.114 g tris(hydroxymethyl) aminomethane in 100 mL milli-Q water, pH 7.4; solution B (0.25 M EDTA): 18.6 g disodium EDTA in 200 mL milli-Q water, pH 8.0. Make up both solution A and solution B with less than total volume of diluent and make up to total volume after pH achieved. Add 6 mL of solution A and 24 mL of solution B to 270 mL milli-Q water.

11. Proteinase K: 5 mg Proteinase K (R&D Systems, Minneapolis, MN) in 100 mL proteinase K buffer (*see Note 4*).

2.5. **Immunohistology**

1. Control tissue (usually human tonsil).
2. Humidified chamber: Large plastic container with sealable lid, damp strips of absorbent paper in bottom, and two glass rods for slides to rest on.
3. Coplin jars.
4. 1% Bovine serum albumin in PBS.
5. PBS.
6. Hydrophobic pen (DAKO).
7. Primary antibody.
8. Biotinylated secondary antibody.
9. Streptavidin–horseradish peroxidase (DAKO).
10. Liquid diaminobenzidine (DAB) substrate–chromogen system (DAKO).
11. Staining jars containing Mayer’s hematoxylin, Scott’s bluing solution, alcohol (90% and absolute), and xylene.
12. DPX mounting medium.
13. Glass coverslips.
14. Microscope eyepiece with counting grid.

3. Methods

To examine the immune response to oral organisms in detail, both the local (gingival tissues and to some extent GCF) and systemic (serum, peripheral mononuclear cells) immune responses are of interest. The following techniques comprise a set of assays which would be useful in dissecting out these local and systemic responses in human patients. All may be adapted for use in animal models. Commercially available kits provide an easier although possibly more expensive alternative to the ELISA method described here (2). Multiplexed particle-based immunoassays read by a flow cytometer are available (BD) and are able to measure six analytes in a single sample. The advantage of this is that sample volume is greatly reduced compared with conventional ELISAs while the sensitivity of the assay is maintained.

3.1. Sample Processing

3.1.1. Peripheral Blood

1. Blood should be processed as soon as possible but can be left at room temperature for up to 8 h (do not refrigerate).
2. Spin blood collected in potassium EDTA tubes at $800\times g$ for 10 min at room temperature, carefully draw off serum and transfer to labeled tubes. Serum can be stored at -20°C for short-term use (up to 3 months) but for longer term storage, it is best kept at -80°C .
3. Transfer blood collected in sodium heparin tubes to 50-mL Falcon tubes (15-mL blood/tube), then add 15 mL RPMI to each tube. Draw up 20 mL Ficoll in a 20-mL syringe attached to plastic mixing cannula. Place the tip of the cannula at the bottom of the tube and slowly and carefully underlay blood and RPMI with 18 mL of Ficoll, avoiding introduction of air bubbles. Centrifuge for 30 min ($400\times g$) at 18°C . Using a sterile transfer pipette, remove the plasma fraction. Some may be retained for later analysis of antibody or cytokine levels using ELISA. Carefully transfer the buffy coat (contains mononuclear cells) to a fresh 50-mL Falcon tube. Buffy coats from extra tubes of the same sample may be combined into one tube at this stage (**Fig. 20.1**). Wash with RPMI and centrifuge at $630\times g$ for 10 min at 18°C . Resuspend the pellet in 5 mL of culture medium (*see Section 2.3*). Peripheral blood mononuclear cell suspension can now be stained for cytokine profiling (**Section 3.4**), set up in culture (**Section 3.3**), or stored in DMSO at -80°C (**Section 3.3**).

3.1.2. GCF

1. Sites sampled should be clinically examined and periodontal probing depth and bleeding on probing recorded. To reduce contamination with whole blood, sites with profuse bleeding should be avoided. Isolate the site with cotton rolls and saliva ejector to avoid contamination with saliva. Gently air-dry the site and remove supragingival plaque with a sterile periodontal curette. Insert the Millipore filter strip into the gingival sulcus until mild resistance is felt and leave in place for 30 s. Repeat this procedure three times per site.
2. Immediately after collection, place strips in tubes containing 10 mM NaH_2PO_4 and 150 mM NaCl, pH 7.2. Vortex tubes thoroughly before centrifuging at $800\times g$ for 5–10 min. Transfer supernatants to labeled cryotubes for storage at -80°C until analysis.

3.1.3. Tissues

1. To prepare tissue samples for histology, immerse the specimen in 30% sucrose in PBS at 4°C until it sinks (up to

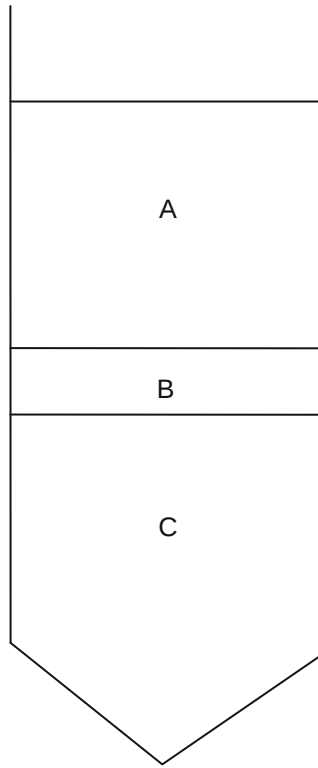


Fig. 20.1. Ficoll fractions of peripheral blood after centrifugation. **(A)** Plasma; **(B)** mononuclear cells; **(C)** red blood cells and Ficoll.

2 days). This will reduce the risk of cracking during freezing and the development of ice crystals within the tissue. Formalin fixation is best avoided if tissues are to be used for immunohistology since formalin may reduce the immunoreactivity of a range of antigens necessitating the use of antigen retrieval methods. Fill a small lidded insulated container with liquid nitrogen. Fashion a foil tray to float on the liquid nitrogen and replace the lid of the container for 10 min. Fill the mould with OCT and gently submerge the tissue in the OCT. Place the mould on the foil tray and replace the lid until OCT is solid. Wrap in foil and label before storing in liquid nitrogen or at -80°C (*see Note 5*). Using a cryostat, cut $5\text{ }\mu\text{m}$ thick sections and mount on glass microscope slides. Allow slides to dry at room temperature for 30 min. Fix in equal parts of acetone and chloroform for 5 min, then dry at room temperature, before wrapping individually in plastic wrap and storing at 4°C .

2. To prepare tissues for T-cell culture, cut the specimen into 1 mm fragments and incubate in digestion medium for 90 min with gentle rolling at 37°C . The ratio of tissue

to medium should be 100 mg:1 mL. Work the fragments through the nylon mesh of the cell strainer to obtain a single-cell suspension. Wash the cells twice with RPMI to remove the collagenase. The suspension should then be centrifuged on a Ficoll-Paque gradient to remove keratinocytes, fibroblasts, and tissue debris. In a 15-mL Falcon tube, add the cell suspension (made up to 6 mL) and 2 mL FCS. Underlay with 6 mL Ficoll (*see* **Section 3.1.1**). Centrifuge for 30 min ($400\times g$) at 18°C. Transfer the buffy coat to another 15-mL Falcon tube, wash in RPMI, then resuspend in 2 mL of culture medium. Set up in culture immediately or leave overnight at 4°C if necessary.

3.2. ELISA

1. Design plate setup (**Fig. 20.2**), allocating wells for standard curve and blanks. Wells may be set up in duplicates or triplicates.
2. Coat all wells except those for standard curve and uncoated blanks with 1 $\mu\text{g/mL}$ antigen in carbonate buffer (100 $\mu\text{L/well}$).
3. Coat standard curve wells with doubling dilutions of human IgG (400, 200, 100, 50, 25, 12.5, 6.25, and 3.13 ng/mL) in carbonate buffer. Add 100 $\mu\text{L/well}$ carbonate buffer to uncoated blank wells.
4. Cover plate with plate sealer and incubate overnight at 4°C.
5. Wash plate three times using 300 μL PBS Tween/well. If plate washer is not available, use a plastic squeeze bottle to fill wells with PBS Tween (avoiding air bubbles), flick well contents into waste container, then invert plate and tap gently onto absorbent paper to remove excess.
6. Block all wells with 1% BSA in PBS Tween (250 $\mu\text{L/well}$) and incubate at room temperature for 1 h. Wash once with PBS Tween.
7. Add serum samples diluted in PBS to appropriate wells (100 $\mu\text{L/well}$) and 100 μL PBS/well to standard curve and blank wells. Incubate at room temperature for 1 h. Wash three times. Use appropriate precautions when washing human samples from well if plate washer is not used (avoid creating aerosol and perform procedure in biohazard hood).
8. Add peroxidase-conjugated anti-human IgG antibody (1:5,000) to all wells (100 $\mu\text{L/well}$) and incubate at room temperature for 1 h. Wash three times.
9. Prepare color substrate solution immediately before use. Per plate: 15 mL citrate buffer, 15 μL of 3% H_2O_2 , 1.5 mL *o*-tolidine. Add 150 $\mu\text{L/well}$. Allow blue color to develop for 10 min. Stop the reaction by adding 3 M HCl

1 A Sample 1	2 →	3 Sample 7	4 →	5 Sample 13	6 →	7 Sample 19	8 →	9 Sample 25	10 →	11 Sample 31	12 →
B Sample 2	→	Sample 8	→	Sample 14	→	Sample 20	→	Sample 26	→	Sample 32	→
C Sample 3	→	Sample 9	→	Sample 15	→	Sample 21	→	Sample 27	→	Sample 33	→
D Sample 4	→	Sample 10	→	Sample 16	→	Sample 22	→	Sample 28	→	Sample 34	→
E Sample 5	→	Sample 11	→	Sample 17	→	Sample 23	→	Sample 29	→	Sample 35	→
F Sample 6	→	Sample 12	→	Sample 18	→	Sample 24	→	Sample 30	→	Sample 36	→
G IgG 400 ng/mL	→	200 ng/mL	→	100 ng/mL	→	50 ng/mL	→	Blank Not coated	→	Blank coated	→
H 25 ng/mL	→	12.5 ng/mL	→	6.25 ng/mL	→	3.13 ng/mL	→	Blank Not coated	→	Blank coated	→

Fig. 20.2. An example of a 96-well ELISA plate setup. Sample wells are in duplicate, allowing analysis of 36 samples per plate. Dilutions of a standard of known concentration are included to allow determination of antibody concentrations in the samples. There are negative control wells for the standard curve (uncoated blanks) and for the samples (coated blanks).

(50 μ L/well). Wells will turn yellow color. *o*-Tolidine is absorbed through the skin, respiratory, and digestive tracts and is toxic and carcinogenic. Refer to the material safety data sheet for details on precautions for use, storage, and disposal.

10. Read plate at 450 and 655 nm to obtain optical density (OD) readings for each well.
11. ODs are proportional to the antigen concentration in the sample. Subtract the OD of the uncoated blanks from the standard curve ODs. Subtract the OD of the coated blanks

from the OD of the sample wells. The readings from the sample wells can now be compared with a standard curve of the known concentration standards to obtain a concentration of the antigen in the sample. The final concentration should correct for the dilution factor of the original sample.

3.3. T-Cell Culture

1. Determine the concentration of viable cells in the suspension prepared in **Section 3.1.3**. Add stock solutions (2 mg/mL) of acridine orange and ethidium bromide together and dilute to 40 µg/mL in PBS. Take 30 µL of the cell suspension and add to an equal volume of the diluted acridine orange and ethidium bromide mixture in a tube. Mix gently, then place a small aliquot beneath the coverslip of a hemocytometer chamber. Count viable cells under fluorescence and calculate the number of viable cells per milliliter in the cell suspension. Acridine orange stains DNA green and ethidium bromide stains DNA orange but is excluded from viable cells. Therefore, viable cells will stain green and non-viable cells will stain orange.
2. Remove half of the cell suspension and make up in culture medium at a concentration of 2×10^6 cells/mL. Add 1 mL to each well of a 24-well plate. Top up each well with a further 1 mL culture medium/well. Add the antigen chosen to stimulate specific T cells present in the culture to a concentration determined to be optimal by pilot studies. We have found that concentrations in culture between 0.5 and 5 µg/mL are suitable when stimulating with *P. gingivalis* outer membrane antigens (5, 14). Incubate in a humidified atmosphere of 5% CO₂ in air at 37°C.
3. Freeze the remaining half of the cell suspension in DMSO. These cells will be irradiated and subsequently used as autologous antigen-presenting cells. DMSO is a cryopreservative, reducing cell damage by partially solubilizing the membrane and inhibiting ice crystal formation (15). The action of DMSO on the cell membrane however will rapidly lead to cell death at room temperature; so it is critical that these steps are performed quickly and on ice. Centrifuge the cell suspension, remove the supernatant, then add a predetermined volume of DMSO. Each cryotube to be frozen should contain $1\text{--}6 \times 10^6$ cells in 0.5 mL DMSO. Gently pipette suspension up and down a few times before aliquoting into labeled cryotubes. Quickly transfer cryotubes to a cryogenic controlled rate freezing container which has been previously cooled in a –80°C freezer and return to the –80°C freezer. The container will control the rate of freezing to around 1°C/min.

4. Change medium three times per week by removing half the volume from each well and replacing with fresh medium.
5. Observe the progress of the cultures using an inverted microscope. Split when cells become confluent (*see Note 6*). Gently pipette medium up and down in well and transfer half to a new well. Top up both wells with fresh medium.
6. Restimulate every 2 weeks by adding specific antigen and irradiated autologous antigen-presenting cells. Quickly thaw a vial of previously frozen autologous cells by pipetting RPMI into the vial and transferring contents to a 15-mL Falcon tube. Do this several times until frozen cells are completely transferred to the Falcon tube and suspended in 15 mL RPMI. Centrifuge for 10 min at 1,500 rpm. Remove the supernatant and make up to 2 mL with culture medium supplemented with 10 U/mL recombinant human IL-2. IL-2 is added to the cultures from this point so that the now specific T cells will proliferate. Perform a viability count. Irradiate the cells in the tube using gamma irradiation (30 Gray), then add 1×10^6 irradiated cells to each of the culture wells, along with the stimulating antigen at the previously determined concentration.
7. Analyze cells using flow-cytometric staining techniques described below after two restimulations, that is, after 5 weeks in culture. Cells not used for staining can be frozen down in DMSO for subsequent use.

3.4. Flow Cytometry

This method can be used to stain T cells from lines established in culture or fresh PBMNCs. Mononuclear cells from mouse spleens can also be collected, separated by Ficoll, and stained using this protocol with the relevant antibodies.

1. Harvest T-cell lines after 5 weeks in culture as described above or collect PBMNCs after Ficoll-Paque density centrifugation. Centrifuge for 10 min at $400\times g$ and remove supernatant. Make up in a small amount of PBS azide and count to determine concentration of cells. Label polystyrene tubes and transfer approximately 2×10^5 cells in 300 μ L PBS azide into each tube.
2. Add antibody to cell surface antigen. The optimal volume of antibody to be added should be determined by previous trials. We use 4 μ L FITC-conjugated mouse anti-human CD4 or CD8 (Pharmingen, San Diego, CA). Pipette the antibody onto the side of each appropriate tube, then vortex thoroughly. If performing double-membrane staining, add the second antibody to the tube now and vortex. Incubate for 30 min at room temperature. Proceed to the washing and fixing stages described below.

3. If the second stain is intracytoplasmic, after incubating with the first cell surface stain, centrifuge at $630\times g$ for 5 min, remove the supernatant using a vacuum aspirator. This should be done carefully so as to remove the most supernatant possible without aspirating the cells. Fix cells by adding 200 μL paraformaldehyde PBS, vortex, then incubate for 5 min and no longer before centrifuging at $630\times g$ for 5 min and aspirating the supernatant without delay.
4. To permeabilize cells, add 200 μL proteinase K buffer, then 50 μL proteinase K and vortex. Incubate at 37°C for 10 min, centrifuge at $630\times g$ for 5 min, and aspirate the supernatant.
5. Add 200 μL PBS azide to each tube followed by the appropriate intracytoplasmic staining antibody. We use 1 μL PE-conjugated rat anti-human IL-4, IFN- γ , IL-10, IP-10, MCP-1, MIP-1 α , and RANTES (Pharmingen) to stain intracytoplasmic cytokines and chemokines. Incubate at room temperature for 30 min.
6. Centrifuge at $630\times g$ for 5 min, aspirate the supernatant. Wash once with 200 μL PBS azide, then add 200 μL FACS fixative.
7. FITC and PE-conjugated specific mouse or rat Ig isotypes (DAKO) should be used as negative controls. We add 0.2 μL of each of these antibodies to control tubes.
8. We analyze 1×10^4 (10,000) cells from each sample using dual-color flow cytometry on a FACSCalibur (Becton Dickinson, Mountain View, CA). The percentage of CD4 and CD8 cells in the samples positive for each antigen can then be determined. Samples are best read soon after staining but can be kept for 3–4 days at 4°C .

3.5. *Immunohistology*

1. Retrieve previously prepared frozen sections from freezer and remove plastic wrap. Obtain sections of human tonsil for use as a positive control. A negative control should also be used and is the sample tissue stained with an irrelevant antibody. Label the slides according to the stain to be used and circle sections using a hydrophobic pen. This will later aid in localizing the antibodies and substrate to the section.
2. Block binding of antibody to nonspecific sites by incubating with 1% BSA in PBS in a coplin jar for 10 min and then washing by immersing in another coplin jar with PBS.
3. Make up the appropriate dilution for the antibody to be used. This will need to be predetermined using a range of dilutions and by selecting the optimal and most

economic dilution. We use a 1:20 dilution for the following primary monoclonal IgG antibodies: mouse anti-human CD4, CD8, CD14, CD19, HLA-DR, CD1a (DAKO), CD25, and CD83 (Pharmingen). The diluted antibody can be applied directly to the section inside the circle made with the hydrophobic pen. The volume required will depend on the size of the section; however, generally 100 μ L is adequate. Incubate the sections in the humidified chamber for 60 min at room temperature.

4. Tap the slide on its side onto absorbent paper to remove the antibody solution, then drop 100 μ L PBS onto section, and tip off to wash. Add 100 μ L of 1:50 biotinylated rabbit anti-mouse immunoglobulins (DAKO) to each section and incubate for 30 min.
5. Wash with PBS, add 100 μ L of streptavidin peroxidase (DAKO), then incubate for 30 min.
6. Wash with PBS, then develop the color reaction with a liquid DAB substrate–chromogen system. Add 1 drop of DAB/mL of the buffer provided, then add 100 μ L of the mixture to each section. DAB is a possible carcinogen, therefore avoid skin contact and work in a fume hood. See material safety data sheet for details of precautions for storage, use, and disposal.
7. Leave color to develop for 10 min, then wash with PBS.
8. Immerse sections in two changes of distilled water for several minutes each.
9. Immerse in Mayer's hematoxylin for 30 s.
10. Wash in distilled water for a few minutes.
11. Immerse in Scott's bluing solution for 30 s, then wash quickly with tap water.
12. Dehydrate by immersing sections in 90% alcohol, then two changes of absolute alcohol for 2 min each, then three changes of xylene for 2 min each.
13. Mount coverslips with DPX while still wet with xylene.
14. Allow to dry overnight in fume hood.
15. Observe the section using a light microscope. The brown-stained areas indicate the presence of the antigen. Compare with the negative control section to determine if the staining is specific. Nuclei will be counterstained blue. To quantify the number of positively staining cells present, use an eyepiece with a counting grid to count all cells and all positive cells in a particular field, then calculate the percentage of positive cells. Select several representative areas and determine the average of these (**Fig. 20.3**).

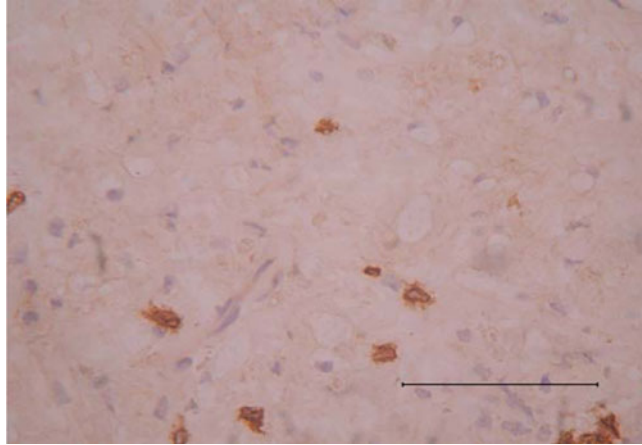


Fig. 20.3. Immunostaining shows CD8+ T cells (*cell surface staining*) of the inflammatory cell infiltrate in sections of a human atherosclerotic plaque (original magnification 400 $\times$). Scale shown represents 100 μ m.

4. Notes

1. If collecting mononuclear cells from peripheral blood, it is possible to save the plasma fraction following Ficoll-Paque density centrifugation for determination of antibody levels by ELISA. However, the dilution factor due to the addition of RPMI 1640 must be taken into account when calculating antibody concentration.
2. Transport medium can be aliquoted into specimen containers and stored at -20°C until required. Ensure medium is defrosted before adding the specimen.
3. Foil cylinders can be constructed in the place of commercially available specimen moulds for embedding tissues for histology. Wrap a small strip of foil several times around the end of a marker approximately 2 cm in diameter (depending on size of tissue). The cylinder should be around 3 cm height.
4. Store all FACS solutions at 4°C and do not use after 3 months.
5. Occasionally, the OCT may crack. If this occurs, defrost the block slowly at 4°C , remove the old OCT, and re-embed.
6. After about 3 days in culture, macrophages and dendritic cells may outnumber the lymphocytes. If they are not removed, the lymphocytes will not proliferate optimally. These cells are around five times the size of the lymphocytes and may have irregular outlines. They will be adherent to the

surface of the well, whereas the lymphocytes are not; therefore if the well is very gently pipetted up and down without disturbing the bottom of the well, the lymphocytes will be able to be transferred to a new well. Wells may need to be pooled if lymphocyte numbers are now too few since they tend to do poorly at low cell concentrations.

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References

1. Ruby, J., and Goldner, M. (2007) Nature of symbiosis in oral disease. *J. Dent. Res.* **86**, 8–11.
2. Orozco, A., Gemmell, E., Bickel, M., and Seymour, G. J. (2006) Interleukin-1 β , interleukin-12 and interleukin-18 levels in gingival fluid and serum of patients with gingivitis and periodontitis. *Oral Microbiol. Immunol.* **21**, 256–260.
3. Katakura, A., Kamiyama, I., Takano, N., Shibahara, T., Muramatsu, T., Ishihara, K., Takagi, R., and Shouno, T. (2007) Comparison of salivary cytokine levels in oral cancer patients and healthy subjects. *Bull. Tokyo Dent. Coll.* **48**, 199–203.
4. Al Amoudi, N., Al Shukairy, H., and Hanno, A. (2007) A comparative study of the secretory IgA immunoglobulins in mothers and children with SECC versus a caries free group children and their mothers. *J. Clin. Pediatr. Dent.* **32**, 53–56.
5. Ford, P. J., Gemmell, E., Walker, P. J., West, M. J., Cullinan, M. P., and Seymour, G. J. (2005) Characterization of heat shock protein – specific T cells in atherosclerosis. *Clin. Diagn. Lab. Immunol.* **12**, 259–267.
6. Gemmell, E., Carter, C. L., and Seymour, G. J. (2004) Mast cells in human periodontal disease. *J. Dent. Res.* **83**, 384–387.
7. Cole, K. L., Seymour, G. J., and Powell, R. N. (1987) Phenotypic and functional analysis of T-cells extracted from chronically inflamed human periodontal tissues. *J. Periodontol.* **58**, 569–573.
8. Gemmell, E., Feldner, B., and Seymour, G. J. (1992) CD45RA and CD45RO positive CD4 cells in human peripheral blood and periodontal disease tissue before and after stimulation with periodontopathic bacteria. *Oral Microbiol. Immunol.* **7**, 84–88.
9. Gemmell, E., Carter, C. L., Hart, D. N., Drysdale, K. E., and Seymour, G. J. (2002) Antigen-presenting cells in human periodontal disease tissues. *Oral Microbiol. Immunol.* **17**, 388–393.
10. Ford, P. J., Gemmell, E., Chan, A., Carter, C. L., Walker, P. J., Bird, P. S., West, M. J., Cullinan, M. P., and Seymour, G. J. (2006) Inflammation, heat shock proteins and periodontal pathogens in atherosclerosis: an immunohistologic study. *Oral Microbiol. Immunol.* **21**, 206–211.
11. Seymour, G. J., Ford, P. J., Cullinan, M. P., Leishman, S., and Yamazaki, K. (2007) Relationship between periodontal infections and systemic disease. *Clin. Microbiol. Infect.* **13**, 3–10.
12. Ford, P. J., Gemmell, E., Timms, P., Chan, A., Preston, F. M., and Seymour, G. J. (2007) Anti-*P. gingivalis* response correlates with atherosclerosis. *J. Dent. Res.* **86**, 35–40.
13. Bird, P. S., Gemmell, E., Polak, B., Paton, R. G., Sosroseno, W., and Seymour,

- G. J. (1995) Protective immunity to *Porphyromonas gingivalis* infection in a murine model. *J. Periodontol.* **66**, 351–362.
14. Gemmell, E., Woodford, V., and Seymour, G. J. (1996) Characterization of T lymphocyte clones derived from *Porphyromonas gingivalis* infected subjects. *J. Periodontal Res.* **31**, 47–56.
15. Mazur, P. (1970) Cryobiology: the freezing of biological systems. *Science*. **168**, 939–949.

Chapter 21

Analysis of Immune Responses to Purified Recombinant Antigens of Periodontal Pathogens

Koichi Tabeta and Kazuhisa Yamazaki

Abstract

The accumulating knowledge about host–pathogen interactions in infectious diseases shows how the immune system interfaces with pathogens, and thus, helps us in understanding the pathogenesis of diseases and improving their treatment. Purified antigens are indispensable while investigating the immune response in both innate and acquired immunities. It is ideal to use native antigens purified from the host organisms in native conditions that sustain their biological activity completely. However, purification of native antigens, especially on a large scale, is technically difficult and generally time consuming. Purifying protein as a peptide-tagged fusion protein is an effective approach. Purification of a recombinant protein engineered to incorporate a polyhistidine tag at either the carboxyl or amino terminus is an established method, and it can be easily modified to obtain optimal results under different conditions. Heat-shock proteins were highly conserved during evolution and are highly homologous between prokaryotic and eukaryotic cells. Their molecular mimicry might have roles in the pathogenesis of chronic inflammatory diseases. We successfully generated histidine-tagged recombinant heat-shock proteins from the periodontopathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. The recombinant proteins allowed us to evaluate the immune response to these antigens in periodontitis patients.

Key words: GroEL, histidine tag, human heat-shock protein 60 (hsp60), recombinant protein expression in *Escherichia coli*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*.

1. Introduction

The method for purification of fusion protein with a histidine tag at either the carboxyl or amino terminus is well established. The affinity of the hexahistidine (6×His) tag for Ni²⁺ allows separation of the fusion protein from bulk bacterial proteins, with up to 95% purity, using a metal chelate affinity column (1, 2). The

fusion protein is prepared by appropriate construction of a plasmid containing the cloned DNA and affinity purification of the protein expressed in various cell expression systems. A researcher can select a plasmid vector and a purification system according to the requirement, from a variety of commercial products available. Heat-shock proteins belong to a family of proteins, which have been conserved during evolution and are highly homologous between prokaryotic and eukaryotic cells. Heat-shock proteins of 60-kDa (hsp60) are strongly immunogenic and immune responses to hsp60 are speculated to have a role in chronic inflammatory diseases through their molecular mimicry (3–5). To analyze the immunogenicity of hsp60 in periodontal pathogens, we purified recombinant GroEL, a homolog of hsp60 from *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (6, 7). We cloned groEL genes into the pRSET expression vector designed for high-level protein expression with at the amino terminus from cloned genes in *Escherichia coli*. The recombinant plasmids were transformed into *E. coli* BL21 (λ DE3) pLysS and then GroEL proteins were expressed as 6 $\times$ His-tagged fusion proteins. GroEL proteins tagged by 6 $\times$ His were purified using a Ni²⁺ chelate affinity column from the solubilized cell lysates (Fig. 21.1). Aliquots of each fraction from the column were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight of the eluted protein coincided with the expected size from the nucleotide

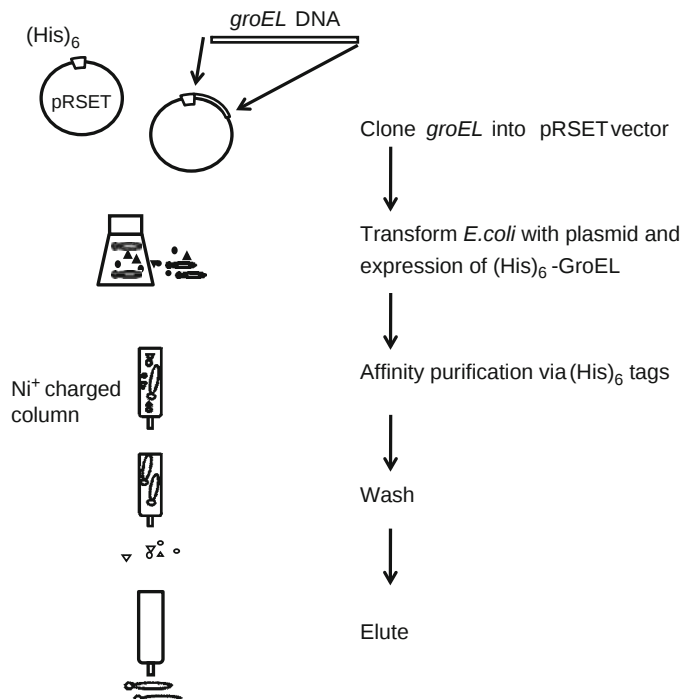


Fig. 21.1. Generation and purification of histidine-tagged GroEL.

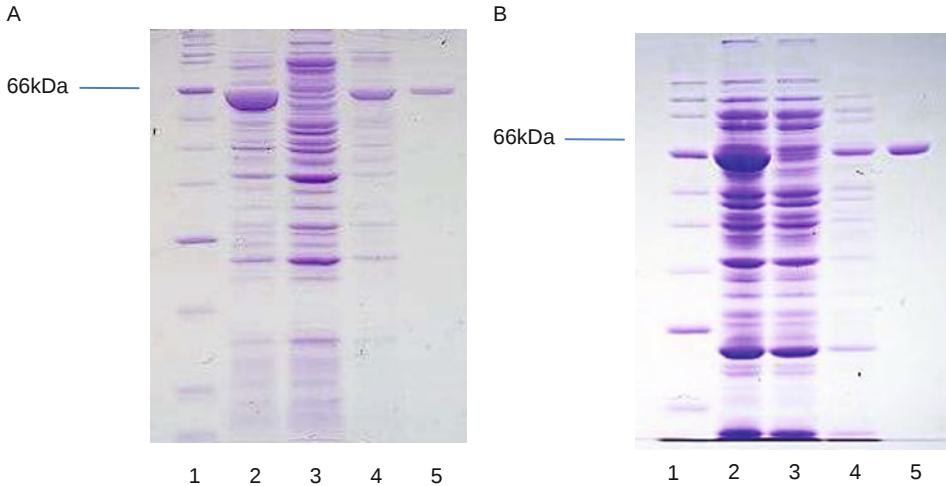


Fig. 21.2. SDS-PAGE analysis of recombinant *Aggregatibacter actinomycetemcomitans* (A) and *Porphyromonas gingivalis* GroEL (B). Polyacrylamide gels (10%) are stained with Coomassie Brilliant Blue. Lane 1, molecular mass marker. Lane 2, *Escherichia coli* lysates transformed with *A. actinomycetemcomitans* GroEL plasmids. Lane 3, flow through fraction from the column. Lane 4, washed fraction from the column with wash buffer containing 60 mM imidazole. Lane 5, fraction eluted with elution buffer containing 500 mM imidazole.

sequence. Gel image analysis revealed a purity of more than 99% (Fig. 21.2). Specificity of the recombinant protein was confirmed by Western blot analysis using anti-pentahistidine monoclonal antibodies and LK-2, which recognizes conserved peptide sequences of bacterial hsp60 (Fig. 21.3). The 6×His-GroEL proteins were used to detect antibodies in the serum of



Fig. 21.3. Western blot analysis of histidine-tagged *Aggregatibacter actinomycetemcomitans* GroEL. SDS-PAGE is used to separate 1 μ g of purified protein, which is then blotted onto the nitrocellulose membrane. The reactivities of anti-hsp60 antibody (LK-1 and LK-2) and anti-pentahistidine antibody are examined. Lane 1, molecular weight marker. Lane 2, reacted with LK-2. Lane 3, reacted with LK-1. Lane 4, reacted with anti-pentahistidine antibody. LK-2 (Reproduced from (7) with permission from Blackwell Publishing Ltd).

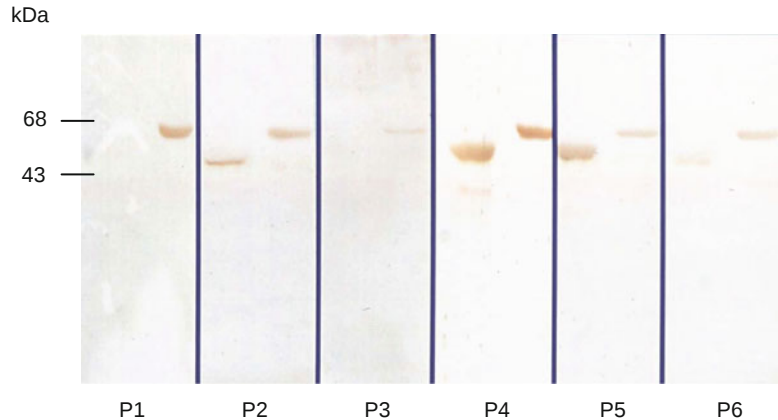


Fig. 21.4. Immunoblotting of purified proteins by serum antibodies from patients. Purified proteins (1 μ g) of recombinant hsp60 (left lane) and *Porphyromonas gingivalis* GroEL (right lane) blotted onto the nitrocellulose membrane were detected by sera from periodontitis patients. The number of patients is indicated (Reproduced from (6) with permission from Wiley-Blackwell).

periodontitis patients using Western blot and ELISA analyses (Fig. 21.4) (6, 7). Furthermore, we used *P. gingivalis* GroEL for the stimulation of antigen-presenting cells and demonstrated clonal expansion of specific T-cells in peripheral blood T-cell pools by single-strand confirmation polymorphism analysis (8, 9). Thus, the purification of recombinant protein is quite simple and beneficial in the analysis of immune responses to diseases. In this chapter, we describe the cloning and purification of recombinant *A. actinomycetemcomitans* GroEL and analysis of immune response to the purified proteins.

2. Materials

2.1. Preparation of Bacterial DNA

1. Todd-Hewitt broth (Difco Laboratories, Detroit, MI) supplemented with 10% horse serum, 1% yeast extract, bacitracin (75 μ g/mL), and vancomycin (5 μ g/mL)
2. Tris-EDTA (TE) buffer supplemented with 0.5% SDS and proteinase K (100 μ g/mL)
3. Ten percentage cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl solution
4. Phenol/chloroform/isoamyl alcohol 25:24:1 (GIBCO BRL, Gaithersburg, MD)

2.2. Cloning the Gene and Preparing the Expression Vector

1. Restriction endonucleases (*Pst*I and *Eco*RI) (Nippon Gene Co., Ltd., Tokyo, Japan)

2. Oligo primer analysis software (National Biosciences, Plymouth, MN)
3. pRSET-A expression vector (Invitrogen, Carlsbad, CA)
4. Qiaquick PCR purification kit (Qiagen, Hilden, Germany)
5. T4 DNA Ligase (Takara Bio Inc., Shiga, Japan)
6. Competent *E. coli* BL21(λ DE3) pLysS
7. Ampicillin: prepare stock solution of 50 mg/mL in deionized water and filter-sterilize using a 0.22 μ m Millipore membrane filter. Final concentration in media is 50 μ g/mL.
8. SOC medium (Invitrogen).
9. LB medium: combine 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 950 mL of deionized water. Stir the solution until completely dissolved. Adjust pH to 7.0 using 5 N NaOH. Adjust volume to 1 L with water. Sterilize by autoclaving (121°C for 20 min).
10. LB agar plates: add 1.5 g of agar to 100 mL of LB medium and autoclave. Cool the molten agar to 50°C. Add 100 μ L ampicillin (final concentration 50 μ g/mL) just before dispensing into petri plates.

2.3. Expression and Purification of Recombinant Protein

1. Isopropyl β -D-1-thiogalactopyranoside (IPTG) stock: 100 mM in deionized water.

2.4. Purification of GroEL by Nickel Chelate Affinity Column

1. Syringe filter: 0.45 μ m HiTrap affinity column: 1 mL (GE Healthcare UK Ltd, Buckinghamshire, UK)
2. Solution of NiSO₄: 0.1 mM NiSO₄ filtered through a 0.45 μ m Millipore membrane filter
3. Binding buffer: 0.02 M Na₂HPO₄, 0.5 M NaCl, 8 M Urea, 10 mM imidazole (pH 7.4)
4. Eluting buffer: 0.02 M Na₂HPO₄, 0.5 M NaCl, 10 mM imidazole (pH 7.4)

2.5. SDS-PAGE

1. 1 $\times$ running gel buffer: 1.5 M Tris-HCl, pH 8.8
2. Thirty percentage acrylamide solution in a dark glass bottle (Wako Pure Chemical Industries, Ltd, Osaka, Japan)
3. Ten percentage SDS (sodium dodecyl sulfate)
4. Ten percentage ammonium persulfate: stock for single use (stored at -20°C)
5. 1 $\times$ stacking gel buffer: 0.5 M Tris-HCl, pH 6.8
6. Running buffer: 0.025 M Tris base, 0.192 M glycine, 0.1% SDS

7. 2× sample buffer: 0.1 M Tris–HCl (pH 6.8), 4% SDS, 6% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue

2.6. Western Blotting

1. 0.45 μm polyvinylidene fluoride (PVDF) membrane or nitrocellulose membrane (GE Healthcare UK Ltd.)
2. Transfer buffer: 0.025 M Tris base, 0.192 M glycine, 20% methanol, 0.1% SDS
3. Blotting cell (Bio-Rad Laboratories; Trans-Blot SD Semi-Dry Transfer cell)
4. Power supply
5. Blocking buffer: Dulbecco's Phosphate Buffered Saline (PBS; Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), 5% milk powder, 0.05% Tween 20
6. Wash buffer: Dulbecco's PBS, 0.5% milk powder, 0.05% Tween 20
7. Primary antibody: LK-1 (StressGen Biotechnologies Corp., Canada), LK-2 (StressGen Biotechnologies Corp.), anti-6×His tag antibody (Qiagen GmbH, Hilden, Germany)
8. Secondary antibody (biotin-labeled anti-mouse IgG) (Vector Laboratories, Burlingame, CA)
9. Vectastain ABC kits (Vector)
10. Diaminobenzidine (DAB) substrate (Wako Pure Chemical Industries, Ltd.)
11. Hydrogen peroxide (H₂O₂) (Wako Pure Chemical Industries, Ltd)

2.7. Immunoblotting of Purified Proteins by Serum Antibodies from Patients

1. Items 1–6 and 9–11 required in **Section 2.6** (Western blotting)
2. Blood sample in a plain plastic blood tube
3. Secondary antibody (biotin-labeled anti-human IgG) (Vector Laboratories, Burlingame, CA)

3. Methods

3.1. Preparation of Bacterial DNA

1. Grow *A. actinomycetemcomitans* Y4 strain in Todd–Hewitt broth at 37°C in a 5% CO₂ atmosphere (*see Note 1*).
2. Harvest cells from 1 mL of turbid culture by centrifugation for 1 min (10,000*g*) and wash twice with sterile distilled water.

3. Resuspend the cells in 567 μL of lysis buffer (TE buffer supplemented with 0.5% SDS and 100 $\mu\text{g}/\text{mL}$ proteinase K) and incubate at 37°C for 1 h.
4. Add 100 μL of 5 M NaCl and 80 μL of CTAB/NaCl solution to complete cell lysis. Incubate at 65°C for 10 min.
5. Add 700 μL of phenol/chloroform/isoamyl alcohol and vortex.
6. Centrifuge for 10 min (6,000*g*).
7. Collect the aqueous (top) phase and add isopropanol (0.6 volumes). Centrifuge for 10 min (10,000*g*).
8. Decant the supernatant carefully and rinse with 70% ethanol. Be careful not to disturb the DNA pellet at the bottom of the tube.
9. Centrifuge for 10 min (10,000*g*).
10. Decant the supernatant carefully, briefly air dry (to allow the ethanol to evaporate completely), and dissolve the pellet in 100 μL of TE buffer.

3.2. Cloning the *groEL* Gene and Preparation of the Expression Vector

1. Amplify the *A. actinomycetemcomitans* *groEL* gene by polymerase chain reaction (PCR) using primers containing recognition sites for the restriction endonucleases *Pst*I and *Eco*RI (5'-AACTGCAGAATGGCAGCAAAAGACGTAA AATTC-3' and 5'-CGGAATTCCGCTGAGCAGGGAGG AATTACATC-3', respectively). The primer sequences are determined based on the sequence data (DDBJ accession number D28817) and Oligo primer analysis software (National Biosciences, Plymouth, MN) (*see* **Note 2**).
2. PCR was performed with Ex Taq DNA polymerase (TaKaRa Bio Inc.) in a final volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , and dNTPmix (0.2 mM each) in an automated DNA thermal cycler. The cycle profile for amplification (32 cycles) is as follows: denaturation at 94°C for 1 min; annealing at 59°C for 1 min; extension at 72°C for 2 min. The last cycle was extended for 5 min.
3. Load the PCR products in a 1% agarose gel and carry out electrophoresis. Excise the *GroEL*-containing DNA fragment in expected size ($\sim 1,600$ kb) on the gel and purify the DNA fragment (*see* **Note 3**).
4. Digest the purified *GroEL*-containing DNA fragment and the prokaryotic expression vector pRSET-A with restriction enzymes *Pst*I and *Eco*RI, respectively (*see* **Note 4**).
5. Ligate the *GroEL* DNA fragment and pRSETA using T4 ligase for 30 min (*see* **Note 5**). The reaction volume

- (20 μ L) for ligation contains GroEL DNA fragments (0.3 pmol) and pRSET A (0.03 pmol) (*see Note 6*).
6. Thaw competent *E. coli* Top10 F' on ice 5 min before adding ligated DNA for transfection (*see Note 7*).
 7. Add 10 μ L of the reaction mixture to the competent *E. coli* cells for ligation. Mix gently and incubate the cells on ice for 30 min.
 8. After incubation, heat shock the cells at 42°C for 45 s. Incubate on ice for 2 min.
 9. Add 1 mL of SOC medium and incubate the culture for 45 min at 37°C with vigorous shaking in a rotary shaker.
 10. Plate 100 μ L of the transformation mixture onto LB agar plates containing ampicillin. Incubate for approximately 16–20 h.
 11. Select the clone carrying the correct sequence of GroEL in the frame with the 6 $\times$ His tags and in the proper orientation by sequencing (*see Note 8*).
 12. After confirmation of the sequence, transform the constructed plasmids into competent cells of *E. coli* BL21(λ DE3) pLysS to facilitate the expression of the recombinant protein (*see Note 9*).

3.3. Expression and Purification of Recombinant Protein

1. Inoculate 10 mL of LB containing 100 μ g/mL ampicillin with a single colony of recombinant *E. coli* BL21 (λ DE3) pLysS carrying the GroEL-containing expression plasmid.
2. Incubate overnight at 37°C with vigorous shaking ($\sim$ 200–250 rpm).
3. Inoculate 1 mL of the overnight culture into 50 mL of LB medium containing 100 μ g/mL ampicillin.
4. Incubate the culture with shaking ($\sim$ 200–250 rpm) at 37°C till it reaches mid-logarithmic phase (*see Note 10*).
5. Add 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) to induce expression of the groEL gene.
6. Grow the culture at 37°C with shaking ($\sim$ 200–250 rpm) for a further 6 h.
7. Harvest cells by centrifugation (3,500*g* for 10 min) and discard the medium (*see Note 11*).

3.4. Purification of GroEL by Nickel Chelate Affinity Chromatography

1. Freeze the cells at -80°C and thaw three times (*see Note 12*).
2. Suspend the cells in 30 mL of binding buffer containing 8 M Urea and incubate at room temperature for 1 h.

3. Centrifuge for 10 min at 10,000*g*. Collect the aqueous phase. Be careful not to disturb the debris containing DNA as it may clog the filter in the next step.
4. Filter the supernatant through a 0.45 μm Millipore membrane filter and adjust the pH to 7.4 prior to subjecting it to the HiTrap affinity column.
5. Wash the column with 5 mL of distilled water (*see Note 13*).
6. Load 0.5 mL of 0.1 M NiSO_4 onto the column.
7. Wash the column with 5 mL distilled water.
8. Equilibrate the column with binding buffer.
9. Load the protein sample onto the column.
10. Wash the column with 20 mL of binding buffer.
11. Elute with 10 mL of elution buffer.
12. Analyze each fraction of the sample by SDS-PAGE.

3.5. SDS-PAGE

1. The use of a slab gel (160 mm $\times$ 160 mm, 1 mm thick) is described in this protocol. Other methods for SDS-PAGE are available elsewhere as alternatives.
2. To prepare 10% polyacrylamide gel, mix 4 mL of 1 $\times$ running gel solution with 6.42 mL of distilled water, 5.34 mL of 30% acrylamide solution, 160 μL of 10% SDS, 64 μL of 10% ammonium persulfate, and 13 μL of TEMED. Pour the mixture into the gel space. Overlay with water-saturated isobutanol (*see Note 14*).
3. After polymerization of the running gel ($\sim$ 30 min after pouring), rinse the top of the gel with water, and then prepare the stacking gel. Mix 2 mL of 1 $\times$ stacking gel buffer with 4.8 mL of distilled water, 1,060 μL of 30% acrylamide solution, 160 μL of 10% SDS, 64 μL of 10% ammonium persulfate, and 5 μL of TEMED.
4. Pour the stacking gel solution on top of the solidified running gel and insert the comb. Keep the gel undisturbed for at least 1 h to allow complete polymerization of the gel.
5. Assemble the electrophoresis unit (gel and chamber). Pour the running buffer into the top chamber and check that it does not leak out of the top chamber, and then fill the lower chamber.
6. Remove the comb and purge wells with a syringe loaded with the running buffer. This is to remove any unpolymerized gel.
7. Load the samples ($\sim$ 1–5 μg) (prepared in sample buffer) (*see Note 15*).

8. Run the gel by passing an initial constant current of 20 mA when the samples are in the stacking gel and then increasing the current to 50 mA when the samples enter the running gel.
9. Switch off the current when the dye front reaches the end and carefully peel the gel off the glass plate.
10. Soak the gel in either transfer buffer for Western blotting or in CBB solution for staining.

3.6. Western Blotting

1. Place the filter paper and PVDF membrane cut to the size of the running gel in the transferring buffer for 20 min (*see Note 16*).
2. Place the filter paper on the platform of the blotting cell. Make a stack by placing the PVDF membrane onto the filter paper followed by the gel and then the filter paper again in order to make a stack. Mount the safety lid and cell.
3. Transfer proteins onto the membrane by passing a constant current of 180 mA for 30 min. Wash the membrane in wash buffer (*see Note 17*).
4. Incubate the PVDF membrane at room temperature in the blocking buffer for 2 h. Wash the membrane twice with wash buffer for 10 min. Cut the membrane for carrying out further incubations with each antibody.
5. Add the primary antibody (LK-1, LK-2, or anti-6×His antibody, all diluted 1:1,000) in the blocking buffer and incubate for 1 h. Wash twice for 10 min with wash buffer (*see Note 18*).
6. Add the secondary antibody diluted in blocking buffer and incubate for 1 h. Wash with wash buffer three times (10 min each).
7. Add ABC reaction solution and incubate for 30 min. Wash thrice for 10 min with wash buffer. During incubation, prepare solutions A and B on ice for the next step.
8. Add H₂O₂ to solution B and mix it with solution A. Vortex immediately.
9. A color develops when the detection solution is added to the membrane. The color should be visualized within 2 min. Stop color development by washing with deionized water or addition of 1 M H₂SO₄.

3.7. Immunoblotting of Purified Proteins by Serum Antibodies from Patients

1. Centrifuge the blood tube for 10 min (1,200–1,500*g*) at room temperature. Collect the serum in aqueous phase (*see Note 19*).

2. Perform the SDS-PAGE (**Section 3.5**) and Western blotting (Steps 1–4 of **Section 3.6**).
3. Add serum (diluted 1:100) in the blocking buffer and incubate for 1 h. Wash twice for 10 min with wash buffer (*see Note 20*).
4. Add the secondary antibody (biotin-labeled anti-human IgG) diluted in blocking buffer and incubate for 1 h. Wash with wash buffer three times (10 min each).
5. Perform Western blotting (Steps 7–9 of **Section 3.6**).

4. Notes

1. An alternative to the method described is to use a commercial kit for bacterial DNA isolation.
2. After designing the forward and reverse primers for amplifying the *groEL* gene, the restriction endonuclease recognition sequence was incorporated at the 5'-end of each primer: 5'-AACTGCAGAATG in the forward primer (*Pst*I site underlined) and 5'-CGGAATTCCG in the reverse primer (*Eco*RI site underlined). As the efficiency of restriction cleavage close to the end of DNA fragments is reduced, additional nucleotides at the 5'-terminus are required for each primer. Whereas *Pst*I and *Eco*RI require a minimum of two flanking bases for cleavage, some restriction endonucleases require >5 flanking bases. The additional bases will improve restriction digestion but will reduce slightly the specificity of the primer.
3. DNA extraction kits are commercially available.
4. The supercoiled DNA requires more units of enzyme for complete digestion than lambda DNA. See the technical references for the product.
5. Use commercial T4 ligase supplied with the buffer. The reaction takes ~30 min.
6. A water bath set at 55°C should be ready for transfection before ligation is completed.
7. Use the manufacturer's protocol.
8. Purify plasmid DNA from each colony of bacteria using a commercially available plasmid miniprep kit, e.g., QiaPrep supplied by Qiagen. Sequence the plasmid (using the T7 sequencing primer) to confirm that the gene of interest (e.g., *GroEL*) is in-frame and in the correct orientation. Maintain the recombinant plasmid in a suitable *E. coli* strain such as Top10F'.

9. Transform *E. coli* BL21(λ DE3) pLysS cells with the verified plasmid DNA using Steps 6–10 of **Section 3.2**.
10. Incubate for 2.5 h ($OD_{600} \sim 0.5$).
11. Each recombinant protein has different characteristics that may affect optimal expression.
12. Cells can be stored at -80°C if they are not to be used immediately.
13. Use a 10 mL syringe or a peristaltic pump for larger scale purifications.
14. Water-saturated isobutanol is prepared by shaking equal volumes of water and isobutanol in a glass bottle.
15. Add an equal volume of $2\times$ sample buffer to the sample. Incubate at 95°C for 4 min.
16. Nitrocellulose membrane can substitute the PVDF membrane.
17. A current is $\sim 1.5\text{--}2\text{ mA/cm}^2$ for 30 min for transferring.
18. Steps 6 and 7 can be omitted for color development of anti- $6\times\text{His}$ antibody (Qiagen 34530) because horse radish peroxidase is already conjugated.
19. After collecting blood sample, the blood tube needs to be placed for 30 min at room temperature to allow complete coagulation of the blood prior to centrifugation.
20. Here, the primary antibody is the participant's anti-GroEL antibody present in the serum. The biotin-labeled anti-mouse IgG in **Section 3.6** is replaced by the biotin-labeled anti-human IgG in the next step (Step 4 of **Section 3.7**) to detect human antibodies.

Acknowledgments

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References

1. Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R., and Stuber, D. (1988) Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology*. **6**, 1321–1325.
2. Hengen, P. (1995) Purification of His-Tag fusion proteins from *Escherichia coli*. *Trends Biochem. Sci.* **20**, 285–286.

3. Ford, P. J., Gemmell, E., Hamlet, S. M., Hasan, A., Walker, P. J., West, M. J., Cullinan, M. P., and Seymour, G. J. (2005) Cross-reactivity of GroEL antibodies with human heat shock protein 60 and quantification of pathogens in atherosclerosis. *Oral Microbiol. Immunol.* **20**, 296–302.
4. Kiessling, R., Gronberg, A., Ivanyi, J., Söderström, K., Ferm, M., Kleinau, S., Nilsson, E., and Klareskog, L. (1991) Role of hsp60 during autoimmune and bacterial inflammation. *Immunol. Rev.* **121**, 91–111.
5. Maeda, H., Miyamoto, M., Kokeguchi, S., Kono, T., Nishimura, F., Takashiba, S., and Murayama, Y. (2000) Epitope mapping of heat shock protein 60 (GroEL) from *Porphyromonas gingivalis*. *FEMS Immunol. Med. Microbiol.* **28**, 219–224.
6. Tabeta, K., Yamazaki, K., Hotokezaka, H., Yoshie, H., and Hara, K. (2000) Elevated humoral immune response to heat shock protein 60 (hsp60) family in periodontitis patients. *Clin. Exp. Immunol.* **120**, 285–293.
7. Tabeta, K., Yoshie, H., and Yamazaki, K. (2001) Characterization of serum antibody to Actinobacillus actinomycetemcomitans GroEL-like protein in periodontitis patients and healthy subjects. *Oral Microbiol. Immunol.* **16**, 290–295.
8. Yamazaki, K., Ohsawa, Y., Itoh, H., Ueki, K., Tabeta, K., Oda, T., Nakajima, T., Yoshie, H., Saito, S., Oguma, F., Kodama, M., Aizawa, Y., and Seymour, G. J. (2004) T-cell clonality to *Porphyromonas gingivalis* and human heat shock protein 60s in patients with atherosclerosis and periodontitis. *Oral Microbiol. Immunol.* **19**, 160–167.
9. Yamazaki, K., Ohsawa, Y., Tabeta, K., Ito, H., Ueki, K., Oda, T., Yoshie, H., and Seymour, G. J. (2002) Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. *Infect. Immun.* **70**, 2492–2501.

Chapter 22

Single-Strand Conformation Polymorphism Analysis for the Diagnosis of T-Cell Clonality in Periodontal Disease

Kazuhisa Yamazaki and Harue Ito

Abstract

T cells recognize antigens via the T-cell receptor (TCR). Diversity in antigen recognition by T cells is generated in part by the recombination of V, (D), J, and C segments of the TCR. It is further enhanced by the N region, in addition to non-germline-encoded nucleotides at the V-(D)-J junction. It is generally believed that each T cell bears a distinct clonotype of TCR and that each clonotype is responsible for an antigen-specific T-cell response. T-cell clonal expansion has been detected in the peripheral blood or the disease-affected sites in patients with infections, autoimmune diseases, malignancy, and post-transplantation complications. Since antigen stimulation of T cells induces the proliferation of specific T cells, clonal T-cell expansion is considered to be a result of an antigen-specific immune response. For the analysis of such antigen-specific T cells, it is common to use their specific antigens if they are known. However, there are many diseases, such as periodontal diseases, in which there are a number of putative pathogenic antigens involved. In these circumstances, the detection of clonally expanded T cells is an effective method to evaluate whether antigen-specific immune responses are involved, since only a few clonally expanded T cells are detected in healthy individuals. In addition, the characterization of any clonally expanded T cells that are detected would further promote the understanding of the disease mechanisms. By using single-strand conformation polymorphism (SSCP) analysis, we demonstrated that oligoclonal T-cell accumulation was present in periodontitis lesions, in contrast to a heterogeneous T-cell population in the peripheral blood. SSCP is a powerful tool for analyzing specific T-cell responses both in vitro and in vivo.

Key words: T-cell receptor β -chain, reverse transcriptase-polymerase chain reaction (RT-PCR), single-strand conformation polymorphism (SSCP).

1. Introduction

T cells are important in the regulation of many aspects of infectious and autoimmune diseases. It is well established that T cells are present in the lesional inflammatory infiltrates of periodontal

diseases (1) and identification of the T-cell populations that mediate the immune responses to periodontopathic bacteria is central to the understanding of periodontal disease. Cell-mediated responses are often dominated by T cells expressing a particular V β or V α gene segment (2). There are several reports of attempts to identify clonotypes by means of monoclonal antibodies (mAb) that react with each of the T-cell receptor (TCR) molecules (3, 4). However, with these approaches, it is still difficult to detect an individual clone within a heterogeneous T-cell population, because the availability of these antibodies is limited. Although investigators have tried to establish specific T-cell clones in vitro, these do not reflect the actual kinetics in vivo (5).

A novel method of overcoming these problems employs a combination of reverse transcriptase-polymerase chain reaction (RT-PCR) with multiple TCR-specific primers and subsequent single-strand conformation polymorphism (SSCP) (6–8). This procedure entails the extraction of total RNA from the tissues of interest, conversion of the RNA to complementary DNA (cDNA), polymerase chain reaction (PCR)-mediated amplification of the TCR V β -C β lesion, gel electrophoresis as SSCP, and, finally, the detection of polymorphisms. This procedure is able to detect not only the accumulation of different T-cell clonotypes in the lesion, but also the expansion of certain T-cell clonotypes in vitro upon stimulation with particular antigens (9) (Fig. 22.1).

In previous studies, we have clearly demonstrated that the T cells infiltrating lesions recognize only a restricted number of antigens or epitopes (10–12) (Fig. 22.2). Furthermore, we have demonstrated that SSCP can detect clonal expansion of specific T cells in peripheral blood T-cell pools by stimulation with outer membrane proteins or heat shock protein (GroEL) from *Porphyromonas gingivalis*, a representative periodontopathic bacteria (4, 13, 14) (Fig. 22.3).

In addition, we have investigated the proportion of the invariant V α 24J α Q TCR within the V α 24 T-cell population in periodontitis and gingivitis lesions using SSCP methodology. Natural killer (NK) T cells were identified with a specific J α Q probe, whereas the total V α 24 TCR was identified using an internal C α probe. NK T cells were a significant proportion of the total V α 24

Fig. 22.1. (continued) SSCP analysis can detect certain T-cell clonotypes within a heterogeneous population in terms of differences of nucleotide sequences in the CDR3 region. Total RNA from an inflamed gingival tissue sample was subjected to reverse transcription polymerase chain reaction (RT-PCR) using V β -specific primers and a common C β primer. Agarose gel electrophoresis gave single bands for each V β family. However, the PCR products include gene transcripts derived from a number of different clones using the same V β gene but having different CDR3 (Panel A). By using SSCP technique, these different clones can be separated as a number of distinct bands on polyacrylamide gel (Panel B) (Reproduced from 9 with permission from Wiley–Blackwell).

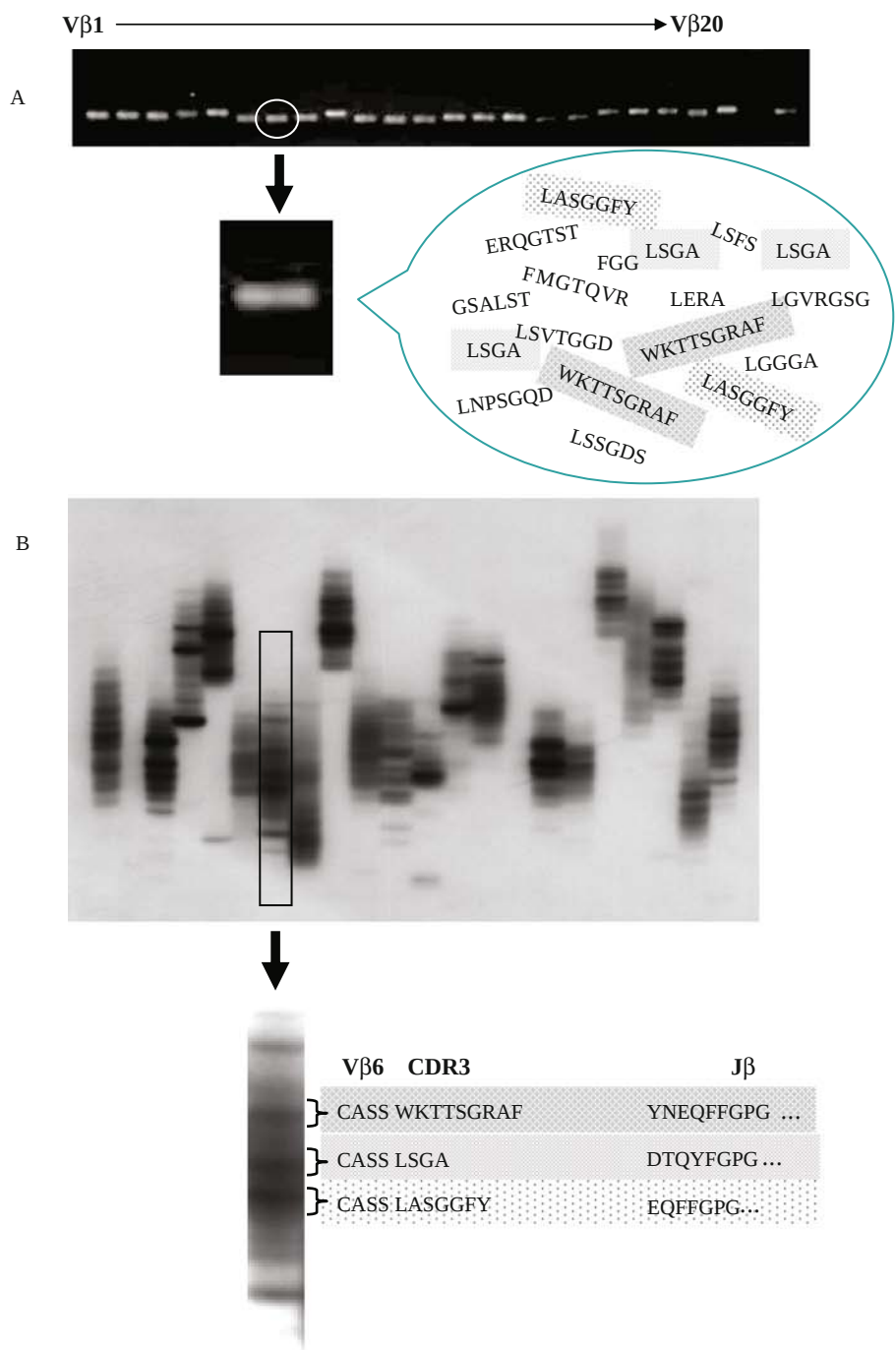


Fig. 22.1. (continued)

population in periodontitis lesions and to a lesser extent in gingivitis lesions, but not in the peripheral blood of either group (15) (Fig. 22.4).

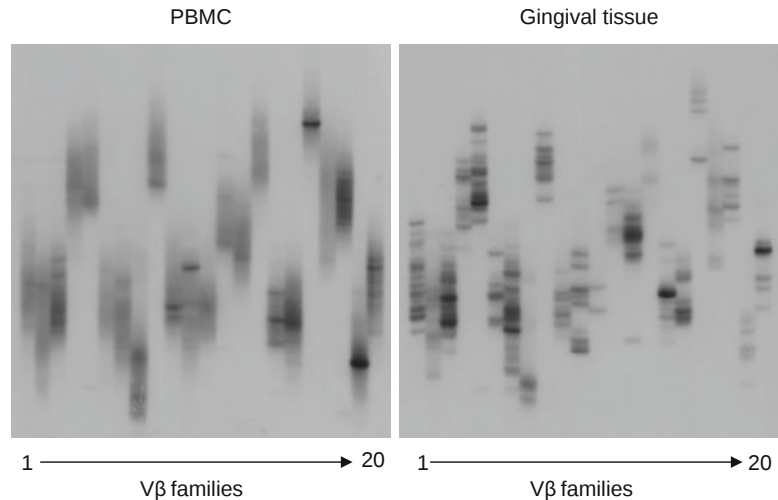


Fig. 22.2. Marked differences in clonality between PBMC (*left panel*) and periodontitis tissue infiltrating T cells (*right panel*) of a patient as identified by RT-PCR-SSCP analysis. PBMC were mostly a smear in most of the V β families, whereas several distinct bands were found in periodontitis lesions.

Thus, SSCP analysis is a powerful tool for investigating specific T-cell responses by detecting not only the static differences of the T-cell clonality between either the different lesions or the lesions and healthy site, but also dynamic responses of T cells to the antigenic stimulation. In this chapter, we present the SSCP procedure in detail.

2. Materials

2.1. Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

1. M-MLV reverse transcriptase kit (usually includes 5 $\times$ or 10 $\times$ reaction buffer).
2. 0.1 M Dithiothreitol.
3. RNase inhibitor (10 U/mL).
4. Random hexanucleotides (50 μ M).
5. β -actin mix primer (Forward and reverse; as internal control).
6. *Taq* DNA polymerase-based PCR kit (usually includes 10 $\times$ reaction buffer).
7. Deoxynucleotide triphosphate (dNTP) mixture comprising dATP, dCTP, dGTP, and dTTP (usually 10 mM each but dependent on the supplier).
8. TCR-specific primers (*see* **Table 22.1**).

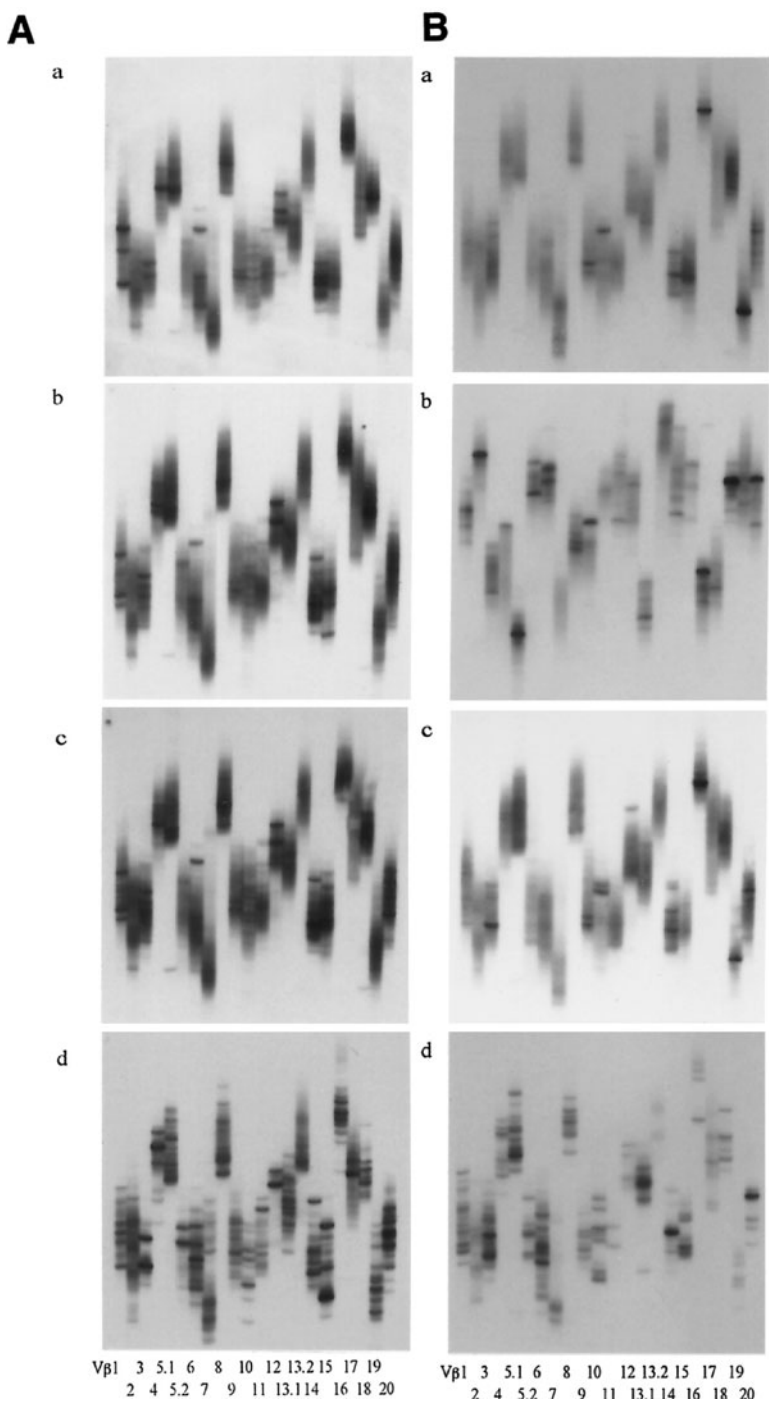


Fig. 22.3. T-cell clonalities of PBMC with or without stimulation with either human heat shock protein 60 (hsp60) or *P. gingivalis* GroEL in a control subject (A) and a periodontitis patient (B). Gingival tissue of periodontitis lesions from the periodontitis patient was analyzed simultaneously. PCR products encoding TCR V β genes from PBMC and gingival tissue were analyzed by the SSCP method as described in **Sections 2 and 3**. **A** Unstimulated PBMC; **B** Human hsp60-stimulated PBMC; **C** *P. gingivalis*-stimulated PBMC; **D** Gingival tissue. Each lane represents a particular V β family (Reproduced from 14 with permission from the American Society for Microbiology).

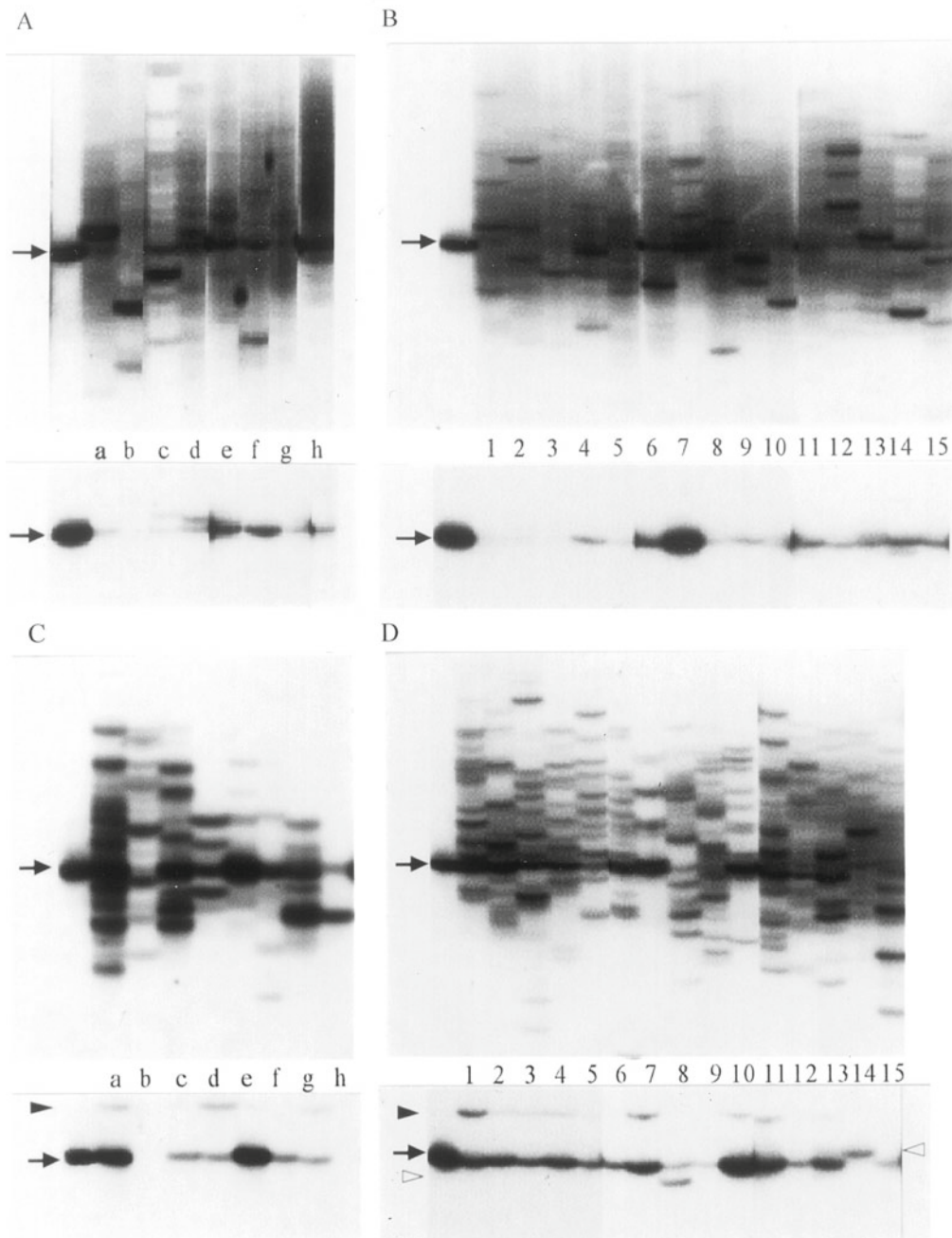


Fig. 22.4. Demonstration of the V α 24 T-cell clonalities and the invariant V α 24J α Q TCR in peripheral blood of controls (A), peripheral blood of periodontitis patients (B), gingival tissue of controls (C), and gingival tissue of periodontitis patients (D). Total RNA was extracted from PBMC and gingival tissues and then subjected to RT-PCR-SSCP analysis. The *top* and *bottom panels* indicate the total V α 24 TCR SSCP profiles as detected with the C α probe and the invariant V α 24J α Q band as detected with the J α Q-specific probe, respectively. The cloned V α 24J α Q DNA fragment as a positive control was applied as shown in the far *left lanes*. The alphabetical code and the numerical code correspond to each control (A and C) and each patient (B and D), respectively, implying that blood samples and gingival tissues were taken from the same subjects and patients. *Arrows* indicate the position for the invariant V α 24 J α Q TCR. The unique bands are indicated by the *arrowheads*. Another unique band which appeared in patients 8 and 14 is indicated by the open *arrowhead* (Reproduced from 15 with permission from The American Society for Investigative Pathology).

Table 22.1
Primer sequences for RT-PCR

V β gene family	Primer sequence (5'3')
V β 1	GCACAACAGTTCCCTGACTTGCAC
V β 2	TCATCAACCATGCAAGCCTGACCT
V β 3	GTCTCTTAGAGAGAAGAAGGAGCGC
V β 4	ACATATGAGAGTGGATTTGTCAATT
V β 5.1	ATACTTCAGTGAGACACAGAGAAAC
V β 5.2	TTCCCTAACTATAGCTCTGAGCTG
V β 6	AGGCCTGAGGGATCCGTCTC
V β 7	CCTGAATGCCCCAACAGCTCTC
V β 8	ATTTACTTTAACAACAACGTTCCG
V β 9	CCTAAATCTCCAGACAAAAGCTCAC
V β 10	CTCCAAAACTCATCCTGTACCTT
V β 11	TCAACAGTCTCCAGAATAAGGACG
V β 12	AAAGGAGAAGTCTCAGAT
V β 13.1	CAAGGAGAAGTCCCCAAT
V β 13.2	GGTGAGGGTACAACCTGCC
V β 14	GTCTCTCGAAAAGAGAAGAGGAAT
V β 15	AGTGTCTCTCGACAGGCACAGGCT
V β 16	AAAGAGTCTAAACAGGATGAGTCC
V β 17	CAGATAGTAAATGACTTTTCAG
V β 18	GATGAGTCAGGAATGCCAAAGGAA
V β 19	CAATGCCCCAAGAACGCACCCTGC
V β 20	AGCTCTGAGGTGCCCCAGAATCTC
C β	TTCTGATGGCTCAAACAC
Biotinylated C β probe	GTGTTTGAGCCATCAGAA

2.2. Polyacrylamide Gel

1. Separating buffer (10 $\times$): 0.5 M Tris-HCl, pH 8.3, 2 mM ethylenediamine tetraacetic acid (EDTA) disodium salt dehydrate and 1 M boric acid (TBE) (*see Note 1*).
2. Ammonium persulfate: prepare 10% solution in water, dispense into single use (200 μ L) aliquots and immediately freeze at -20°C (*see Note 2*).
3. 4% acrylamide/bis solution (49:1), 1 $\times$ TBE, 5% glycerol *N,N,N,N'*-Tetramethyl-ethylenediamine (TEMED, Bio-Rad, Hercules, CA), and 0.25% APS.
4. Running buffer (0.5 $\times$): Dilute 10 $\times$ TBE with double-distilled water for use.

5. Loading dye: Mixture of 10 mL of formamide, 10 mg of xylene cyanol, 10 mg of bromophenol blue and 200 μ L of 0.5 M EDTA.

2.3. Southern Blotting for TCR Gene

1. Nylon membrane (Immobilon-Ny; Millipore Intertech, Bedford, MA).
2. Biotinylated C β probe (*see* **Table 22.1** for the nucleotide sequence).
3. Hybridization bag.
4. SSPE, pH 7.4 (20 $\times$): 3 M NaCl, 0.2 M NaH₂PO₄·2H₂O, 20 mM Na₂ $\times$ EDTA (adjust to pH 7.4 with NaOH if necessary). The solution is autoclaved (121°C for 20 min) and stored at room temperature.
5. Denhardt's solution (100 $\times$): Prepare 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) bovine serum albumin (fraction V) in autoclaved double-distilled water. Filter using a 0.45 μ m membrane. Store at -20°C.
6. Hybridization solution: 10% (v/v) 20 $\times$ SSPE, 5% (v/v) 100 $\times$ Denhardt's solution, 0.5% (w/v) SDS. Store at room temperature.
7. Blocking buffer: 25 mM phosphate buffer (pH 7.2), 125 mM NaCl, 5% (w/v) SDS. Prepare at 40–50°C. Filter using a 0.45 μ m membrane. Store at room temperature.
8. Wash buffer I: Dilute blocking buffer (1 volume) in 9 volumes of double-distilled water. Store at room temperature.
9. 10 $\times$ Wash buffer II: 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM MgCl₂. Filter using a 0.45 μ m membrane filter. Store at 4°C (*see* **Note 3**).
10. Enhanced Chemiluminescence (ECL) reagent (Phototope[®]-Star Detection Kit, New England Biolabs, Inc., Beverly, MA).
11. X-ray film and corresponding developing fluid and fixing solution (RX-U; Fuji Photo Film Co, Ltd., Tokyo, Japan).

3. Methods

3.1. Extraction of Total RNA from Peripheral Blood Mononuclear Cells (PBMC) and Gingival Tissues and the PCR Reaction

1. Separate the mononuclear cells (PBMC) by Ficoll-Paque density gradient centrifugation from 10 mL of autologous peripheral blood (refer to **Chapter 20**, this volume).
2. Finely dice approximately 100 mg of the gingival tissues in an RNA extraction solution such as Trizol (*see* **Note 4**).
3. The first strand cDNA is synthesized using M-MLV reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and 50 μ M

random hexanucleotides (Takara Shuzo, Shiga, Japan) from 2 μg of total RNA in the supplied reaction buffer (GIBCO BRL) containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , supplemented with 0.5 U RNase inhibitor (Invitrogen Co, San Diego, CA), 0.1 M dithiothreitol (Invitrogen), and dNTP (each at 0.5 mM) (Takara). The reaction mixture is incubated at 37°C for 60 min and then heated at 95°C for 5 min to inactivate the enzyme.

4. Utilizing the 22 V β family-specific 5' primers coupled with one biotinylated-C β 3' primer designed by Choi et al. (14), PCR was performed with 2.5 units of *Taq* DNA polymerase (Takara) in a final volume of 15 μL containing 6 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% gelatin, and dNTP (each at 0.2 mM) in an automated DNA thermal cycler (Takara). The amplification parameters were as follows: initial denaturation at 94°C for 7 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min; final extension at 72°C for 7 min (see **Note 5**).

3.2. SSCP

1. Setting up the gel mold (glass plate sandwich): Take one plain glass plate (40 cm $\times$ 20 cm) and one notched glass plate from the drying racks to the Acrylamide Gel Pouring Area (see **Note 6**).
2. Wipe the inside surface of the plates. Treat the notched glass plate only with Sigmacote (Sigma Aldrich, St. Louis, MO), then wipe dry both plates with deionized water, and then with 95% ethanol. Then wipe two plastic spacer strips with ethanol and place one along each of the long edges of the long plate. Place the notched plate directly on top with the inside surface down, and make sure the plates and spacers are lined up fitting snugly against the lower edge of both plates.
3. Place four clamps along one long side of the glass plate sandwich, leaving about 1" unclamped at the bottom (see **Note 7**). Carefully place one long strip of sequence gel tape along the bottom edge of the sandwich and approximately 12.7 cm (5") up the side edges. Make sure the tape is sealed tightly along the bottom and sides by firmly pressing it along the edges of the glass (the depth) before folding it and sealing along the length and width of the glass. Put a fifth clamp on each side of the sandwich near the bottom (on the taped area) (**Fig. 22.5**).
4. Prepare 40 mL of 4% gel as indicated in **Section 2.2**. Dispense the gel solution carefully using an automated pipettor with a 20 mL pipette so as not to form air bubbles. Slide the plastic combs in between the plates into the gel

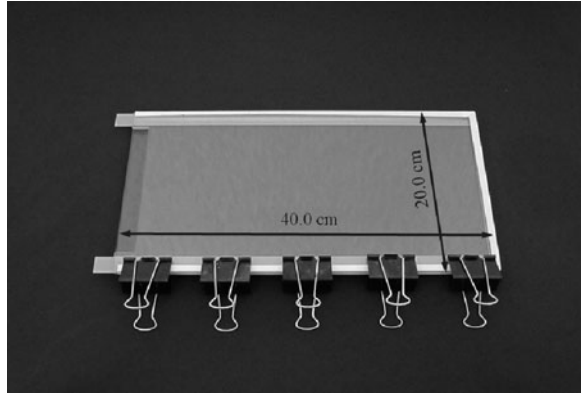


Fig. 22.5. Setting up the gel mold. Note the position of clamps.

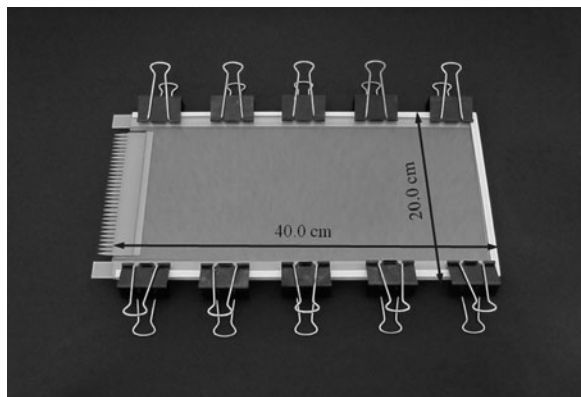


Fig. 22.6. Photo of the completed gel mold. Slide the plastic combs in between the plates into the gel mix with the flat sides in (i.e., the shark teeth facing outward).

mix with the flat sides in (i.e., the shark teeth facing outward). The flat surfaces of the combs should form an even line, about 0.5 cm deep into the gel (the top of the oblong holes should be even with the top of the short glass plate) (**Fig. 22.6**).

The gel should polymerize in approximately 120 min (*see Note 8*).

5. Remove the paper adhesive tape from the gel plates and set up the electrophoresis apparatus using the clamps. Take two clamps and place them along the top of the gel mold. Spread the three remaining clamps along each side evenly.
6. Add the running buffer ($0.5\times$ TBE) to the upper and the lower chambers of the gel unit. Remove the plastic combs carefully so as not to disrupt the surface of the gel. The remnants of the gel should be removed from the teeth of the comb. Carefully slide the toothed side of the plastic combs into the top of the gel such that the tips of the teeth protrude only slightly into the gel (roughly 0.5–1 mm). If

the comb slides in too easily, the wells will leak, so keep trying the combs until two fit snugly (*see Note 9*).

7. Add approximately 500 mL of water to the jacket of the gel unit connected to the cooling unit (Circulation Type Handy Cooler, Thomas Kagaku Co, Ltd., Tokyo, Japan). Set the temperature at 20°C.
8. Complete the assembly of the gel unit and connect it to a power supply (Electrophoresis Power Supply EPS 3501 XL, General Electric Co., Fairfield, CT).
9. Dilute the amplified DNA in a denaturing solution (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) (1:3 ratio) and maintain at 90°C for 2 min. Load 1–3 μL of each reaction into the wells if using fine-toothed combs, 3–5 μL if using larger toothed combs.
10. Run the gel at 35 W constant power for 130 min.

3.3. Southern Blotting

1. After electrophoresis, the notched glass is carefully removed. Place the membrane (Immobilon-Ny; Millipore Intertech, Bedford, MA) which has been presoaked in running buffer for at least 20 min onto the gel and transfer for 30 min (*see Note 10*).
2. Mark the reverse surface of the membrane and peel off the membrane while dropping running buffer between the membrane and gel. Desiccate the membrane at 60°C for 10 min in the dryer. Crosslink the DNA to the membrane using the UV cross linker (UVP Ultraviolet Crosslinker CL-1000, Ultra-Violet Products, Ltd., Cambridge, UK) at 33,000 $\mu\text{J}/\text{cm}^2$ (at 254 nm) (*see Note 11*).

3.4. Hybridization

1. Put the membrane in a plastic hybridization bag one size larger and add hybridization solution and seal using a heat sealer. Stroke the surface of the bag with the side of a plastic pipette so as to make the solution cover the entire surface of the membrane (*see Note 12*). The bag is then incubated at 42°C for 2 h with gentle shaking.
2. Prepare the biotinylated C β probe during the prehybridization step. The stock solution that had been prepared at 1 pmol/ μL is diluted 1,000-fold (10 μL stock solution in 10 mL of hybridization solution). Discard the hybridization solution from the bag and add 10 mL of the probe solution. Incubate overnight at 42°C with gentle shaking.
3. On the following day, prepare 0.2 $\times$ SSPE/0.5% (w/v) SDS by diluting 20 $\times$ SSPE and 5% (w/v) SDS with double-distilled water. The hybridized membrane is transferred to a new hybridization bag and the 0.2 $\times$ SSPE/0.5% (w/v) SDS solution is added to

the bag (*see* **Note 13**). Incubate the bag in a water bath set at 48°C for 10 min (*see* **Note 14**).

4. Discard the 0.2× SSPE/0.5% (w/v) SDS solution. The hybridized membrane is transferred to a new hybridization bag and 20 mL of blocking solution is added to the bag. Incubate the bag at room temperature for 5 min with gentle shaking.
5. Prepare Wash I and Wash II solutions. Discard the blocking solution (0.2× SSPE/0.5% (w/v) SDS) and add 90 mL of Wash I to the bag. Incubate for 10 min at room temperature with gentle shaking. Discard Wash I and repeat this process again.
6. Prepare streptavidin solution, biotinylated alkaline phosphatase solution, and CDP-Star according to the manufacturer's instructions (Phototope Star detection kit; New England Biolabs, Inc., Beverly, MA). Add streptavidin solution to the bag and stroke the surface of the bag with the side of a pipette at room temperature for 5 min. Discard the streptavidin solution followed by two 5-min washes of the membrane with Wash I solution.
7. Add the biotinylated alkaline phosphatase solution and stroke the surface of the bag with the side of a pipette at room temperature for 5 min. Wash the membrane twice (5 min each) with Wash II solution.
8. Add SDP-Star and stroke the surface of the bag with the side of a pipette at room temperature for 10 min followed by shaking for 5 min. Discard the solution and seal the bag.
9. Clean the surface of the bag with an anionic detergent such as Alconox™ (Alconox, Inc., White plains, NY), followed by 99% EtOH.
10. Set the sealed bag in a film cassette with Scotch tape and expose to X-ray film for 30 s –5 min at room temperature (*see* **Note 15**). The film is developed and fixed (*see* **Note 16**). The typical appearance of T-cell clonality in a periodontitis lesion and peripheral blood pool in the same patient is shown in **Fig. 22.2**.

4. Notes

1. Boric acid should be added after the complete solution of Tris-HCl and EDTA. The buffer is then autoclaved (121°C for 20 min).
2. Before adding TEMED and ammonium persulfate, the gel solution should be filtered using a 0.45 μm membrane

filter. The gel solution should be kept on ice when adding TEMED and APS.

3. MgCl_2 should be added after pH adjustment.
4. The tissues can be stored at -80°C for several weeks if the procedure cannot be carried out immediately.
5. If electrophoresis cannot be carried out on the same day as the PCR, then store the PCR products at -20°C until required.
6. Use always same side of the surface for the pouring space. Place the plates on the table with the inside surfaces of the plates facing up (the inside surface can be identified by marking the outside surface of plate with an indelible marker pen).
7. The pressure points of the clamps should be directly over the center of the spacers.
8. Bubbles can usually be avoided by increasing the tilt angle to the side or more vertically when pouring the gel solution.
9. Once the tips of the shark teeth have been inserted into the gel surface, never pull them up again. The resultant air gaps may result in cross-well contamination.
10. The long and short edges of the membrane ($15\text{ cm} \times 12\text{ cm}$) are in contact with those of the gel plates. Place the membrane to the center in a crosswise direction and the upper edge of the membrane should be 2.5 cm from the upper side of the marker line of xylene cyanol lengthwise. Place a filter paper onto the membrane, and then three paper towel sheets are further placed on the filter paper. Place the removed glass plate onto the paper towel and weigh the glass plate down with a $>500\text{ g}$ weight (e.g., with a product catalog).
11. If the hybridization reaction cannot be carried out immediately, the membrane can be stored in a hybridization bag in the dark at room temperature.
12. Prepare hybridization solution at $1\text{ mL}/10\text{ cm}^2$. Let the air out of the bag as much as possible.
13. Use 100 mL per 200 cm^2 of the membrane.
14. The temperature and the duration should be accurately followed.
15. Exposure time may vary depending on the intensity of hybridization.
16. The developing and fixing solutions should be matched to the type of X-ray film used.

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References

1. Seymour, G. J. (1991) Importance of the host response in the periodontium. *J. Clin. Periodontol.* **18**, 421–426.
2. Matis, L. A. (1990) The molecular basis of T-cell specificity. *Annu. Rev. Immunol.* **8**, 65–82.
3. Mathur, A., Michalowicz, B., Yang, C., and Aeppli, D. (1995) Influence of periodontal bacteria and disease status on V beta expression in T cells. *J. Periodontal. Res.* **30**, 369–373.
4. Nakajima, T., Yamazaki, K., and Hara, K. (1996) Biased T cell receptor V gene usage in tissues with periodontal disease. *J. Periodontal. Res.* **31**, 2–10.
5. Wassenaar, A., Reinhardus, C., Thepen, T., Abraham-Inpijn, L., and Kievits, F. (1995) Cloning, characterization, and antigen specificity of T-lymphocyte subsets extracted from gingival tissue of chronic adult periodontitis patients. *Infect. Immun.* **63**, 2147–2153.
6. Masuko, K., Kato, T., Ikeda, Y., Okubo, M., Mizushima, Y., Nishioka, K. et al. (1994) Dynamic changes of accumulated T cell clonotypes during antigenic stimulation in vivo and in vitro. *Int. Immunol.* **6**, 1959–1966.
7. Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K., and Sekiya, T. (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. USA.* **86**, 2766–2770.
8. Yamamoto, K., Sakoda, H., Nakajima, T., Kato, T., Okubo, M., Dohi, M. et al. (1992) Accumulation of multiple T cell clonotypes in the synovial lesions of patients with rheumatoid arthritis revealed by a novel clonality analysis. *Int. Immunol.* **4**, 1219–1223.
9. Yamazaki, K., and Nakajima, T. (2004) Antigen specificity and T-cell clonality in periodontal disease. *Periodontol.* **2000**, **35**, 75–100.
10. Itoh, H., Ohsawa, Y., Yoshie, H., and Yamazaki, K. (2002) Oligoclonal accumulations of T-cell clones in gingivitis and periodontitis lesions. *Oral Microbiol. Immunol.* **17**, 324–329.
11. Yamazaki, K., Nakajima, T., Ohsawa, Y., Tabeta, K., Yoshie, H., Sakurai, K. et al. (2000) Selective expansion of T cells in gingival lesions of patients with chronic inflammatory periodontal disease. *Clin. Exp. Immunol.* **120**, 154–161.
12. Ohsawa, Y., Yamazaki, K., Nakajima, T., and Hara, K. (2000) Clonal accumulation of T cells bearing V beta 6 T-cell receptor in chronic inflammatory periodontal disease. *Oral. Microbiol. Immunol.* **15**, 211–217.
13. Nakajima, T., Yamazaki, K., Sakurai, K., Gemmell, E., Seymour, G. J., and Hara, K. (1998) Detection of clonotypic changes of T cells after stimulation with *Porphyromonas gingivalis*. *Oral Microbiol. Immunol.* **13**, 238–245.
14. Yamazaki, K., Ohsawa, Y., Tabeta, K., Ito, H., Ueki, K., Oda, T. et al. (2002) Accumulation of human heat shock protein 60-reactive T cells in the gingival tissues of periodontitis patients. *Infect. Immun.* **70**, 2492–2501.
15. Yamazaki, K., Ohsawa, Y., and Yoshie, H. (2001) Elevated proportion of natural killer T cells in periodontitis lesions: a common feature of chronic inflammatory diseases. *Am. J. Pathol.* **158**, 1391–1398.

Chapter 23

Real-Time PCR Focused-Gene Array Profiling of Gingival and Periodontal Ligament Fibroblasts

Patty Chou and Trudy J. Milne

Abstract

The techniques for the establishment of primary gingival and periodontal ligament fibroblast cultures have been well established for over 30 years. It is only more recently, with the commercial availability of real-time PCR (RT-PCR) gene arrays that the expression profiles of up to 84 genes can be carried out simultaneously. Each focused panel of genes can identify the up- or down-regulation of genes associated with any one of over 100 biological pathways or specific disease states. Fibroblasts for RNA extraction and subsequent gene expression analysis can be collected under various experimental conditions and stored in RNA-preserving solution (e.g., *RNAlater*[®]) for processing at a later date or extracted immediately. The “gold standard” method for the extraction of RNA from fibroblasts for RT-PCR purposes is the TRIzol[®] reagent method. With the addition of a spin-column clean-up step, any phenol carried over from the TRIzol[®] step is removed, thus ensuring a high yield of quality RNA. The RNA is then reverse transcribed to cDNA and analyzed using the RT-PCR focused-gene arrays. Data analysis is made easy using on-line array analysis software packages.

Key words: Gingival fibroblast, periodontal ligament fibroblast, primary cell culture, TRIzol[®]-mediated RNA purification, real-time PCR, focused gene array.

1. Introduction

Normal fibroblast function is critical for the maintenance of periodontal tissues and for optimal wound healing responses. Fibroblasts are responsible for extracellular matrix homeostasis and the production of growth factors to facilitate healing. They are the predominant cell type in healthy periodontal connective tissue (1). Although the morphology and growth rate of gingival and periodontal ligament fibroblasts are similar (2), both types of

fibroblasts appear to be heterogeneous *in vivo* (3) and display distinct functional activities in the regeneration and repair of the periodontal tissues (4,5). Melcher (6) first postulated that periodontal ligament fibroblasts had the slowest rate of repopulating the root surface after periodontal surgery, when compared with cells of other periodontal connective tissues. *In vitro*, gingival fibroblasts demonstrate a faster rate of proliferation and wound-fill than periodontal ligament fibroblasts (7). Furthermore, both types of fibroblasts display different gene expression patterns, which may reflect intrinsic functional differences of the two cell populations (8).

While the protocols for *in vitro* gingival and periodontal ligament fibroblast experiments have been established over 30 years, this chapter describes a detailed protocol for use in conjunction with focused-gene array profiling. Standardization of the laboratory procedure is necessary in order to minimize technical errors throughout the experiment. A standardized protocol for fibroblast culture, RNA isolation, and focused-gene array profiling techniques will also allow comparison among different studies.

2. Materials

2.1. Tissue Culture

1. Dulbecco's Modified Eagle Medium (D-MEM) High Glucose (1×) with GlutaMAXTM-I and sodium pyruvate (InvitrogenTM) supplemented with 10% fetal bovine serum (FBS), antibiotic-antimycotic reagent (100×, add 5–500 mL D-MEM) (contains 10,000 units penicillin, 10,000 µg streptomycin, 25 µg/mL amphotericin B) (InvitrogenTM), and gentamicin reagent solution (10 mg/mL), add 2.5–442 mL D-MEM (InvitrogenTM).
2. Phosphate-buffered saline (PBS) autoclaved at 121°C for 25 min.
3. 0.25% Trypsin/0.04% ethylenediamine-tetraacetic acid (EDTA), pH 7.2 (1×) (InvitrogenTM).
4. Plasticware: 25, 75, and 175 cm² Filter Cap Tissue Culture Flasks, 50 mL PP-test tube (Cellstar[®], Greiner Bio-One GmbH), 24-well multidish plate (NuncTM Delta).
5. Carbon dioxide incubator (Heraeus CO₂-Auto-Zero incubator, Germany or equivalent) with a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C.
6. RecoveryTM Cell Culture Freezing Medium (GIBCOTM).
7. CryoTM Freezing tubes (2 mL), (Greiner Bio-One GmbH).
8. Inverted light microscope (PL10/0.25×, Olympus).
9. Hemocytometer.

2.2. RNA Purification

1. RNAlater[®] (Ambion[®], Applied Biosystems).
2. Neptune BT[™] barrier tips (Continental Lab Products).
3. DEPC-water: prepared by adding 1 mL diethylpyrocarbonate (DEPC) to 1 L of deionized (Milli-Q) water. Shake vigorously and incubate overnight in a fume hood. The solution is then autoclaved for 20 min at 121°C to inactivate the remaining DEPC.
4. DNase-, RNase-, and protease-free water (Molecular Biology Grade Water, 5 PRIME Inc.)
5. 70% ethanol (made up to volume with DNase-, RNase-, and protease-free water).
6. TRIzol[®] Plus RNA Purification Kit (Invitrogen[™]).
7. MOPS buffer (10× stock): Prepare by dissolving 3-(*N*-morpholino) propanesulfonic acid (MOPS) 200 mM, 209 MW, 41.8 g/L, sodium acetate (200 mM, 82 MW, 16.4 g/L) and ethylenediamine tetraacetic acid (EDTA) disodium salt (12 mM, 292.24 MW, 3.72 g/L) in DEPC-treated water. The solution is then adjusted to pH 7.0 with 1 M NaOH.
8. RNAway (Molecular BioProducts).
9. 38% formaldehyde solution (BDH).
10. Ethidium bromide (10 mg/mL) (Sigma).
11. RNA Millennium[™] Markers (1 mg/mL) (Ambion, ABI) (optional).
12. NorthernMax[®] Formaldehyde Load Dye (Ambion, ABI).
13. Formaldehyde electrophoresis running buffer: Prepare by diluting 100 mL 10× MOPS stock buffer with 900 mL DEPC-treated water. Mix well.
14. 0.5 M EDTA solution: Prepare by dissolving 186.12 g EDTA (MW 372.24) in 800 mL water. Adjust to pH 8.0 with sodium hydroxide pellets (~20 g) and make up to 1 L with water. The disodium salt of EDTA will only dissolve when the pH of the solution is approximately 8.0.
15. Tris-EDTA (TE) buffer (50× stock): Prepare by dissolving Tris (242 g/L, 2 M, 121 MW) in 500 mL water then add 100 mL 0.5 M EDTA and 57.1 mL glacial acetic acid. Adjust volume to 1 L with distilled water.
16. SYBR[®] Green II RNA gel stain (10,000× concentrate in DMSO) 500 µL (Invitrogen[™]). SYBR Green II stain: Prepare by diluting 20 µL of the stock (10,000×) in 200 mL TE buffer.
17. Spectrophotometer, (e.g., Ultrospec 6300 pro, Amersham Biosciences) or a microvolume spectrophotometer

(NanoVueTM Spectrophotometer [GE Healthcare] or a NanoDropTM [Thermo-Scientific] spectrophotometer).

2.3. Real-Time PCR Focused-Array Gene Profiling

1. RT² First Strand Kit (SABiosciences) (*see Note 1*).
2. RT² SYBR Green/ROX PCR Master mix.
3. RT² ProfilerTM PCR array (qRT-PCR machine and pathway specific).
4. 8-channel pipette 5–50 μ L.
5. Multi-channel pipette reservoir (50 mL) (Corning[®] Costar[®] Reagent Reservoir).

3. Methods

It is important to have an organized plan and be well prepared prior to the tissue collection procedure. Fibroblast culturing is achieved successfully and easily by minimizing the tissue exposure time to unfavorable environments (air, lower temperatures, and lack of nutrients). Prompt preparation and placement of the tissue explant into the culture medium and incubator at 37°C improve fibroblast survival. Equipment and solutions should be sterile and an aseptic technique should be used.

The extracted tooth and gingival tissue are washed twice in freshly prepared culture medium using the technique described by van der Pauw et al. (9) as this minimizes cross contamination between the periodontal ligament and gingival tissues. The extracted tooth and gingival tissue are separated and placed in culture medium. The tooth and gingival explants are subsequently prepared for fibroblast dissemination using the method outlined by Somerman et al. (2). Fibroblasts are cultured using a modified version of the cultivation process described by Owens (10).

The RNA isolation protocol is based on the extraction procedure described by Chomczynski and Sacchi (11). Total RNA is extracted from the fibroblasts according to the multi-step TRIzol[®] Reagent protocol (*see Note 2*).

3.1. Primary Fibroblast Culture

All tissue culture procedures are performed in a laminar flow cabinet (AIRPURE[®]™ Biological Safety Cabinet Class II or equivalent).

3.1.1. Collection of Gingival Tissues and PDL Tissues

1. A 2 × 5 mm strip of keratinized gingival tissue is harvested from the buccal surface of the flap raised to expose the impacted tooth. The gingival explant is placed immediately into a 50 mL PP-test tube containing 35 mL of 37°C D-MEM culture medium.

2. Unerupted third molar(s) are collected and placed into 50 mL PP-test tube(s) containing 35 mL of 37°C D-MEM culture medium (*see* **Note 3**).

3.1.2. Dissemination of Fibroblasts

3.1.2.1. Tooth Explant

1. The tooth explant is removed from the PP-test tube using dental tweezers and the crown of the tooth is held with sterile gauze while the PDL tissue attached to the middle third of the root explant is removed using a scalpel blade or a Columbia 4L-4R universal curette.
2. The PDL tissue is placed into the center four wells of a roughened 24-well multidish plate (*see* **Note 4**). D-MEM culture medium (1 mL) is added into each of the four wells containing the PDL tissues (*see* **Note 5**).
3. The plate is then placed in a humidified 37°C, 5% CO₂ incubator and culture medium replaced every third day.

3.1.2.2. Gingival Explant

1. The harvested gingival explants are cut into small pieces and divided into four equal portions. Each portion is placed into one of the four center wells of a 24-well multidish plate and observed for attachment to the well surface. Culture medium (2–3 mL) is pipetted into each of the four center wells and care is taken not to dislodge the attached gingival tissue.

3.1.3. Separation of Fibroblasts

1. Monitor fibroblast confluence with an inverted light microscope. It may take anywhere from 8 to 24 days for the fibroblasts to reach 80% confluence.
2. When the fibroblasts surrounding the attached explant reach 80% confluence (passage zero [P₀]), the tissue explant is discarded and the fibroblasts are lifted with trypsin/EDTA solution (200 µL/well) and incubated at 37°C for 2 min or until all cells detach from the surface of the well. This is determined by inspecting each well under an inverted light microscope.
3. The trypsin is neutralized by adjusting the pH with D-MEM culture medium (300 µL/well). The suspended cells are transferred to a 25 cm² flask containing D-MEM culture medium (5 mL). The flask is then placed in the humidified 37°C 5% CO₂ (in air) incubator.
4. The culture medium is changed every third day by discarding the old culture medium, washing the cells with PBS (10 mL) and adding 5 mL of freshly prepared culture medium.
5. The cells in the 25 cm² flask are designated as passage one (P₁) cells.

3.1.4. Fibroblast Culture

1. P₁ cells in the 25 cm² flask are grown to 80% confluence (*see Note 6*), then trypsinized (with 3 mL trypsin following the procedure described in **Section 3.1.3**) and transferred into two 75 cm² tissue culture flasks containing 20 mL of D-MEM culture medium and incubated in a humidified 37°C, 5% CO₂ incubator until confluent. The medium is changed every 3 days. These cells in the 75 cm² flask are designated as the passage two (P₂) cells.
2. When confluent, the cells in the 75 cm² flasks are trypsinized and transferred into two 175 cm² flasks. These cells are designated as the passage three (P₃) cells.
3. This process of passing and subculturing (splitting) the cells is continued until the required number of fibroblasts for the experiment is attained.
4. A hemocytometer is used to determine the cell density (*see Note 7*). The cell density of a confluent 175 cm² flask is approximately 2×10^7 cells/mL.
5. At this point, fibroblasts from one 175 cm² flask are collected for cryopreservation and the fibroblasts in the remaining flasks are used for experimentation.

3.1.5. Cryopreservation

1. The fibroblasts from one confluent 175 cm² flask are trypsinized, neutralized with D-MEM media and transferred into a 50 mL PP-test tube. The cell suspension is then centrifuged at 148*g* for 5 min at room temperature (*see Note 8*).
2. The supernatant is removed and the cell pellet is washed by resuspending the cells in PBS and centrifuging again at 148*g* for 5 min at room temperature.
3. The supernatant is removed and the cells are resuspended in the recommended volume (1×10^6 – 10^7 cells/mL) of 4°C RecoveryTM Cell Culture Freezing Medium (*see Note 9*). The homogeneous cell suspension is then dispensed into cryopreservation vials (1 or 1.5 mL).
4. Cryopreservation should be commenced immediately by placing the cryovials into a freezing apparatus, such as a “Mr Frosty” (*see Note 10*) or a polystyrene box with cotton wool, which is then placed in a –80°C freezer. The cryovials can then be transferred to a liquid nitrogen storage vessel after 24 h for long-term storage.

3.2. RNA Purification

3.2.1. Cell Harvesting

1. Cells to be harvested are trypsinized. An inverted microscope is used to confirm the fibroblasts are detached. The cell suspensions from one experimental plate (12-well plate)

are then combined and transferred into a 50 mL PP-test tube that contains 12 mL of D-MEM culture medium.

2. The fibroblast suspension is then centrifuged at $148g$ for 5 min at room temperature. The supernatant is removed and the cell pellet resuspended in 1 mL of RNA *later*[®] and stored in the -80°C freezer until RNA isolation.

3.2.2. RNA Isolation

The TRIzol[®] Plus RNA Purification protocol is outlined in the manufacturer's manual, briefly (*see Note 11*):

1. The fibroblasts suspended in RNA *later*[®] are defrosted on ice and the cell suspension is transferred to a 1.5 mL (eppendorf) tube. The eppendorf tube is centrifuged for 2 min at $12,000g$ at 4°C to pellet the cells.
2. The supernatant is removed and 500 μL TRIzol[®] reagent is added to lyse the cells and achieve a homogeneous sample.
3. The RNA phase separation is achieved by adding 100 μL of chloroform, followed by centrifugation at $12,000g$ for 15 min at 4°C .
4. Following centrifugation a colorless upper aqueous phase is obtained (*see Note 12*). This layer, which contains the RNA, is transferred to a new eppendorf tube.
5. An equal volume of 70% ethanol (in DNase-, RNase-, and protease-free water) is added to obtain a final ethanol concentration of 35%. The sample is then transferred to the Spin Cartridge and the bound RNA washed.
6. The bound RNA is then eluted from the column with DNase-, RNase-, and protease-free water (*see Note 13*).

3.2.2.1. DNase I Treatment (Optional)

1. RNA samples are then treated with Amplification Grade DNase I or TURBOTMDNase (*see Note 14*) according to the manufacturer's instructions.

3.2.3. Assessment of RNA Yield and Purity

This can be done using a spectrophotometer or by formaldehyde agarose electrophoresis. It is good practice to confirm one with the other.

3.2.3.1. Spectrophotometry

Absorbance values should lie between 0.1 and 1.0 to ensure an optimal measurement. The concentration of RNA = $1 A_{260}$ Unit of ssRNA = 40 $\mu\text{g}/\text{mL}$ H_2O . Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1 in a 10 mM Tris buffer. Do not interchange between spectrophotometers as values are machine dependent.

3.2.3.2. Formaldehyde Agarose Electrophoresis

1. To make one formaldehyde agarose gel (1.2%, 30 mL), agarose (0.36 g) is dissolved in DEPC-treated water (26.1 mL) containing $10\times$ MOPS buffer (3 mL) by heating slowly in a microwave (*see Note 15*).

2. When cool (55°C), formaldehyde (0.9 mL) is added and when cool the mixture is poured into an agarose gel tray (*see Note 16*). The gel can be placed at 4°C to speed up the gel-setting process.
3. The sample RNA (200–500 ng) or RNA standard (2 µL, 1 mg/mL) is mixed with 3 volumes of formaldehyde gel loading dye and heated to 65°C for 10 min.
4. Once set, the formaldehyde gel is placed in the electrophoresis tank and 1× MOPS running buffer is added. The RNA samples are loaded into the gel and run at 80 V for approximately 1 h or until the bromophenol blue has migrated almost two-thirds of the gel (*see Note 17*).
5. Stain gel with SYBR Green II for 20 min and visualize stained RNA with a UV transilluminator.

3.3. Real-Time PCR Focused-Array Gene Profiling

3.3.1. cDNA Synthesis

1. Reverse transcribe total RNA (100–1,000 ng) (*see Note 18*) into cDNA using the RT² First Strand Kit as per the manufacturer's protocol. See briefly below.
2. Any remaining genomic DNA is eliminated from the total RNA by mixing 8 µL of total RNA with 8 µL of the 5× gDNA Elimination Buffer. The reaction is incubated at 42°C for 5 min and chilled on ice for 1 min.
3. To each 10-µL genomic DNA-free RNA mixture, 10 µL of a RT Cocktail (4 µL 5× RT Buffer 3, 1 µL Primer and External Control Mix, 2 µL RT Enzyme Mix 3, and 3 µL DNase-, RNase-, and protease-free water) is added and incubated at 42°C for exactly 15 min.
4. Heat the reaction mixture at 95°C for 5 min to stop the reaction. Add 91 µL DNase-, RNase-, and protease-free water. Store at –20°C overnight or at –80°C for long-term storage.

3.3.2. Performing Real-Time RT-PCR

1. Prepare PCR Cocktail in a 50-mL multi-channel pipette reservoir. Combine RT² SYBR Green/ROX PCR Master mix (1,275 µL) and DNase-, RNase-, and protease-free water (1,173 µL) and 102 µL prepared cDNA template. Mix well but avoid making bubbles. This provides a total volume of 2,550 µL of the PCR Cocktail. Load 25 µL of the PCR cocktail into each well of the PCR array (*see Note 19*).
2. Place the sealed PCR Array into the Real-time PCR instrumentation and run according to the two-step cycling program for your specific instrument (*see Note 20*).

3.3.3. Data Analysis

1. Microsoft Excel spreadsheet data files are uploaded to the SABioscience web site (www.sabiosciences.com) for analysis.

4. Notes

1. The kit is designed and optimized for real-time PCR-based gene expression analysis with SABiosciences RT² ProfilerTM PCR arrays. The kit includes a genomic DNA elimination step. It uses random hexamers and oligo-dT primer for reverse transcription in an unbiased manner. A built-in external RNA control helps monitor reverse transcription efficiency and test for enzyme inhibitors contaminating the RNA sample. The magnesium and nucleotide concentrations and other buffer components are compatible with RT² SYBR Green/ROX PCR Master mixes used with the RT² ProfilersTM PCR arrays.
2. See manufacturer's instructions for a detailed protocol.
3. During the collection of the gingival tissues and PDL tissues, it is important to avoid contamination between the PDL tissues on the third molar and the gingival tissues. Root surface contact during the third molar extraction and bone removal should be minimized. These precautions include avoiding sectioning of the third molar. The collected gingival tissue(s) and third molar(s) are transported immediately to the tissue culture room for culturing.
4. Roughening the well surface assists the attachment of the explanted tissues so that disseminating of the fibroblasts occurs more easily.
5. The explant tissue is covered completely by the culture medium to eliminate direct air contact that may lead to fungal colonization.
6. Prior to each splitting procedure, fibroblasts are grown to 80% confluence. Cell detachment by trypsin may be difficult if the fibroblasts are grown to full confluence, as the extracellular materials produced by confluent fibroblasts may act as a protective layer from the trypsin.
7. See <http://www.cascadebio.com/uploads/File/pdf/hemat.pdf>.
8. Centrifuging at a higher speed may cause cells to lyse.
9. It is crucial to store the cells in the RecoveryTM Cell Culture Freezing Medium (GIBCO-BRLTM) with an optimal range of cell densities (1×10^6 – 1×10^7 cells/mL). A low

cell-revival rate may occur if fibroblasts are stored in a density lower than the range described.

10. “Mr Frosty” (NALGENE Labware) – freezing apparatus – gradual freezing reduces the risk of ice crystal formation and cell damage.
11. The TRIzol[®] Plus RNA Purification Kit combines the “gold standard” TRIzol RNA extraction method followed by a spin column step which removes any trace of phenol that may have been left behind after the Trizol[®] extraction step ensuring a quality of total RNA suitable for RT-PCR.
12. Pipetting technique is crucial. Care should be taken to ensure the pipette tip is located only in the colorless upper aqueous phase and avoid the tip touching the white interphase or the lower red phenol–chloroform phase (containing unwanted DNA and protein). Careful pipetting will reduce genomic DNA contamination.
13. The amount of DNase/RNase-free water added to redissolve the RNA pellet depends on the RNA yield. We recommend dissolving the RNA with minimal DNase-/RNase-free water initially, as it is difficult to re-concentrate the dissolved RNA, especially when RNA yields are low. If the RNA yield is high, additional DNase-, RNase-free water can be added at a later stage.
14. The DNase I treatment eliminates the contaminating genomic DNA from the RNA preparation. The Amplification Grade DNase I is an endonuclease isolated from bovine pancreas that digests single- and double-stranded DNA into oligo- and mono-nucleotides. TURBO[™] DNase is a genetically engineered form of bovine DNase I exhibiting greater catalytic efficiency than conventional DNase I.
15. A 1.2% agarose–formaldehyde gel is prepared using the Bio-Rad submarine gel apparatus. The formaldehyde gel protocol must be performed in a fume hood, as the reagents are toxic.
16. Gel electrophoresis trays and combs should be washed with RNAway[™] to remove any RNase contamination.
17. Formaldehyde loading dye contains bromophenol blue and xylene cyanol which migrate to the same point as double-stranded DNA of 300 and 4,000 bp, respectively, in size in an agarose gel. The length of human 18S rRNA and 28S rRNA is 1,868 and 5,025 bp, respectively. Two hundred to 500 ng of RNA can be observed easily with SYBR Green II.

18. Only a volume of 8 μ L can be added to the cDNA synthesis reaction. Therefore, it is important to have a high yield and quality of RNA for an accurate PCR array result.
19. The recipe provides an excess volume of approximately only 140 μ L. Ensure that each well receives the correct volume as the solution is very viscous. The 25 μ L for the last few wells may have to be dispensed using a single head pipette.
20. The PCR parameters for the SDS software were set up according to the SABiosciences (formerly SuperArray) Instrument-Specific Setup Instructions. The software was used for instrument control, data collection, and advanced data analysis (<http://www.sabiosciences.com>).

References

1. Schroeder, H. E., Munzel-Pedrazzoli, S., and Page, R. (1973) Correlated morphometric and biochemical analysis of gingival tissue in early chronic gingivitis in man. *Arch. Oral Biol.* **18**, 899–923.
2. Somerman, M. J., Archer, S. Y., Imm, G. R., and Foster, R. A. (1988) A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J. Dent. Res.* **67**, 66–70.
3. Lekic, P. C., Pender, N., and McCulloch, C. A. (1997) Is fibroblast heterogeneity relevant to the health, diseases, and treatments of periodontal tissues? *Crit. Rev. Oral Biol. Med.* **8**, 253–268.
4. Nishimura, F., and Terranova, V. P. (1996) Comparative study of the chemotactic responses of periodontal ligament cells and gingival fibroblasts to polypeptide growth factors. *J. Dent. Res.* **75**, 986–992.
5. Giannopoulou, C., and Cimasoni, G. (1996) Functional characteristics of gingival and periodontal ligament fibroblasts. *J. Dent. Res.* **75**, 895–902.
6. Melcher, A. H. (1976) On the repair potential of periodontal tissues. *J. Periodontol.* **47**, 256–260.
7. Lackler, K. P., Cochran, D. L., Hoang, A. M., Takacs, V., and Oates, T. W. (2000) Development of an in vitro wound healing model for periodontal cells. *J. Periodontol.* **71**, 226–237.
8. Han, X., and Amar, S. (2002) Identification of genes differentially expressed in cultured human periodontal ligament fibroblasts vs. human gingival fibroblasts by DNA microarray analysis. *J. Dent. Res.* **81**, 399–405.
9. Van der Pauw, M. T., Van den Bos, T., Everts, V., and Beertsen, W. (2000) Enamel matrix-derived protein stimulates attachment of periodontal ligament fibroblasts and enhances alkaline phosphatase activity and transforming growth factor β 1 release of periodontal ligament and gingival fibroblasts. *J. Periodontol.* **71**, 31–43.
10. Owens, R. B. (1974) Glandular epithelial cells from mice: a method for selective cultivation. *J. Natl. Cancer Inst.* **52**, 1375–1378.
11. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159.

Chapter 24

The Use of Gene Arrays in Deciphering the Pathobiology of Periodontal Diseases

Moritz Kebschull and Panos N. Papapanou

Abstract

Gene expression profiling, i.e., the systematic cataloging of messenger RNA sequences in a cell population, organ, or tissue sample, is a powerful means of generating comprehensive genome-level data sets on complex diseases. We have recently applied a systematic transcriptome-based approach in the study of healthy and diseased gingival tissues, as well in the response of peripheral blood mononuclear cells after periodontal therapy. Our data indicate that both the gingival and the circulating transcriptomes correlate with discernible phenotypic characteristics and may further our understanding of the pathobiology of periodontitis. In this chapter, we outline the laboratory steps required for the processing of gingival tissue and blood samples in view of hybridization with whole-genome microarrays.

Key words: Periodontal disease, gene expression, transcriptome, microarray, gingival, peripheral blood, monocyte, lymphocyte.

1. Introduction

Functional genomics, alternatively termed transcriptomics, encompasses the analyses of patterns of gene expression in cells or tissue samples and their correlation with the underlying biology. Transcriptomes are a powerful means of generating comprehensive genome-level data sets on complex diseases and have provided enormous insights mostly in cancer research (1–3), but also in other conditions such as muscular dystrophy (4), Alzheimer’s disease and dementia (5, 6), rheumatologic disorders (7, 8), and asthma (9, 10). We have recently adopted a transcriptome-based approach in the study of the pathobiology of periodontal

diseases. We examined gingival tissue transcriptomes in healthy and diseased gingival tissues (11) and are currently in the process of expanding our previous work (12) that sought to identify distinct gene expression signatures in chronic and aggressive periodontitis. In another set of studies, we examined whether comprehensive periodontal therapy may induce changes in gene expression of peripheral blood mononuclear cells, focusing on the potential of therapy to promote an anti-atherogenic phenotype (13). We propose that the study of both the gingival tissue and the circulating transcriptome will allow an enhanced understanding of the pathobiology of the periodontal diseases and inform the design of subsequent studies. In this chapter, we provide a detailed description of the laboratory steps necessary in order to process gingival tissue samples and peripheral blood samples in view of hybridization with full-genome microarrays.

2. Materials

2.1. Gingival Tissue Harvesting and Processing

1. *RNAlater* (Ambion, Houston, TX, USA)
2. Homogenizer, e.g., PowerGen 700 (Fisher Scientific, Pittsburgh, PA, USA)
3. *Trizol* Reagent (Invitrogen, Carlsbad, CA, USA)
4. 12 × 75 mm polypropylene tubes, e.g., BD Falcon round-bottom tubes (Becton Dickinson & Co. [BD], Franklin Lakes, NJ, USA)

2.2. Blood Collection

1. Standard phlebotomy set, e.g., *Vacutainer* Safety-Lok Blood Collection Set (BD)
2. *Vacutainer* CPT Cell Preparation Tubes 8 mL (BD)

2.3. Blood Cell Separation

1. Cooled centrifuge with releasable brake, e.g., Centrifuge 5702R (Eppendorf)
2. 50 mL Falcon tubes (Fisher)
3. Phosphate-buffered saline without Ca^{2+} / Mg^{2+} (Mediatech, Manassas, VA, USA)
4. Hemocytometer, e.g., improved Neubauer bright-light (Hausser Scientific, Hersham, PA, USA)
5. MACS separation columns (Miltenyi Biotec, Auburn, CA, USA)
6. MACS multistand (Miltenyi)
7. AutoMACS rinsing solution, pH 7.2 (Miltenyi)

8. MACS microbeads (Miltenyi)
 - (a) CD4 (#120-000-440)
 - (b) CD14 (#120-000-305)

2.4. Extraction of Total RNA

1. Chloroform
2. Cooled microcentrifuge, e.g., Centrifuge 5415R (Eppendorf, Hamburg, Germany)
3. Ethanol, molecular biology grade (99.5%)
4. Glycogen (Invitrogen) adjusted to 5 µg/mL with nuclease-free water (Invitrogen)
5. RNeasy Mini kit (Qiagen, Valencia, CA, USA)
6. Microvolume spectrophotometer, e.g., Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA)

2.5. In Vitro Transcription (IVT) and Biotin Labeling

1. (a) For tissue samples: One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA). (b) For monocytes/lymphocytes: Two-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA)
2. Thermocycler, e.g., TGradient (Biometra, Göttingen, Germany)
3. GeneChip Human Genome U133 Plus 2.0 microarrays (Affymetrix)
4. Access to a microarray core facility for hybridization of the samples to Affymetrix microarrays (ask for site-specific instructions for sample preparation, etc.)

3. Methods

3.1. Gingival Tissue Harvesting and Processing

1. Harvest a tissue sample according to standard clinical protocols; place in 1.5 mL tube (e.g., Eppendorf Biopur Safe-lock) with 1 mL RNAlater (*see Note 1*).
2. Hold at 4°C overnight, subsequently drain and freeze at -80°C until further processing.
3. Transfer the tissue sample to a 12 × 75 mm polypropylene tube filled with 1 mL Trizol reagent (under a fume hood). Subsequently, thoroughly homogenize the tissue (three bursts of 20 s each, *see Notes 2–4*).
4. Divide the homogenized sample into two 2 mL nuclease-free Eppendorf tubes. Freeze instantly one tube at -80°C and keep as “backup”; incubate the other for 5 min (fume hood, on ice). If needed, both samples can be frozen at this point (*see Note 5*).

3.2. Blood Collection

1. Phlebotomize according to standard protocols and sample approximately 8 mL blood each into each of four Vacutainer CPT tubes (*see Note 6*).

3.3. Blood Cell Separation

1. Centrifuge tubes for 15 min at 1,000*g* with centrifuge brake turned off (*see Note 7*).
2. Carefully collect the white layer of peripheral blood mononuclear cells using a 5 mL pipette and place in 50 mL Falcon tube (*see Note 8*).
3. Wash with 50 mL of ice-cold PBS (10 min, 300*g*, 4°C), remove supernatant by aspiration.
4. Wash with 15 mL of ice-cold PBS (10 min, 300*g*, 4°C).
5. Resuspend pellet in 10 mL of ice-cold PBS, count the cells using the hemocytometer.
6. Centrifuge (5 min, 300*g*, 4°C), resuspend in PBS to a density of 10⁷ cells per 80 µL. Keep on ice.
7. Add 20 µL of CD4 (or CD14 beads, respectively) per 80 µL of cell suspension, incubate on ice for 15 min.
8. Wash with 10 mL ice-cold PBS, centrifuge (5 min, 300*g*, 4°C), resuspend in 500 µL of ice-cold PBS.
9. Place MACS column in multistand, wash column twice with 5 mL autoMACS solution.
10. Apply cell suspension to MACS column, wash twice with 5 mL autoMACS, collect flow-through, and label tube as "CD14⁻."
11. Remove MACS column from separator stand, place in 15 mL Falcon tube, and elute twice with 5 mL ice-cold autoMACS using the plunger provided and label tube as "CD14⁺."
12. Using the CD14⁻ cell suspension, proceed accordingly for CD4 (beginning from step 7).
13. Pellet each cell population by centrifugation (10 min, 300*g*, 4°C).
14. Add 0.5 mL of Trizol reagent to pellet, mix well by pipetting. Samples can be stored at -80°C, if needed (*see Note 5*).

3.4. Extraction of Total RNA

1. Add 100 µL of chloroform to the sample (*see Notes 9 and 10*), shake vigorously for approximately 15 s, vortex for 1 min, and incubate on ice for 2 min. Centrifuge (15 min, 13,400*g*, 4°C).
2. Carefully transfer the upper, colorless aqueous phase containing the RNA into a new 1.5 mL tube using a pipette

with a 1 mL tip, add 4 μ L glycogen (5 μ g/mL) and 250 μ L ethanol (*see Note 11*). Mix by shaking for 15 s, incubate for 10 min on ice, and spin (10 min, 13,400*g*, 4°C). From this point on, all work can be carried out on a laboratory bench, ideally devoted to RNA work only. Use of a hood further reduces the risk of contamination with nucleases. Clean workspace and instruments with RNase Zap according to the manufacturer's instructions.

3. Remove supernatant by pouring, wash the white RNA pellet (should be clearly visible) with 500 μ L of 80% ethanol (freshly prepared from 100% ethanol and RNase-free water), and centrifuge (10 min, 5,400*g*, 4°C).
4. Remove supernatant by pipetting, invert the tube to allow the pellet to air-dry for approximately 10 min (*see Notes 12 and 13*).
5. Resuspend the pellet carefully in 100 μ L of RNase-free water (*see Note 14*). The extracted total RNA can be stored at -80°C at this point, if needed.
6. To ensure high quality of the extracted RNA, further purify using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions. To ensure sufficient RNA concentration for subsequent reactions, the volume of RNase-free water used to elute the RNA after the column purification should be based on the type of tissue sampled and the pellet size after the precipitation (e.g., 20 μ L for smaller tissue samples and monocytes/lymphocytes, 40 μ L for larger tissue samples).
7. After purification, measure the quality and quantity of the obtained total RNA by spectrophotometric analysis. Typical yields from larger tissue samples are approximately 80–160 μ g, from smaller tissue samples 20–60 μ g, from monocytes or lymphocytes approximately 100–300 ng. The A_{260}/A_{230} ratio is typically between 1.9 and 2.1 (*see Notes 15–18*). The samples can then be stored at -80°C (*see Note 5*).

3.5. *In Vitro* Transcription (IVT) and Biotin Labeling

1. (a) *For tissue samples:* Use 7.5 μ g of total RNA (concentration ≥ 1.0 μ g/ μ L) in a single round of IVT and biotin labeling reaction, using the Affymetrix One-cycle target labeling assay according to the manufacturer's instructions (*see Note 18*). To obtain a correct concentration of the poly-A RNA controls, perform three serial dilutions (2:38–2:98–2:11.3) (*see Note 19*). (b) *For monocytes and lymphocytes:* Use 100 ng of total RNA in a two-round IVT and biotin labeling reaction using the Affymetrix Two-cycle target labeling assay, according to the manufacturer's instructions (*see Note 18*).

2. Twenty micrograms (20 μg) of labeled cRNA is fragmented and hybridized to Affymetrix microarrays according to the manufacturer's instructions at a specialized core facility.

4. Notes

1. Alternatively, the samples can be snap-frozen in liquid nitrogen chair-side and subsequently directly transferred to -80°C . However, keep in mind that handling of liquid nitrogen poses a safety hazard in a clinical setting. Since RNAlater treatment has proven to reliably preserve the sample RNA, we recommend the use of RNAlater over liquid nitrogen. In addition to keeping the RNA stable for up to several days at ambient temperature, it also allows shipment of tissue samples from different study centers to a central processing center.
2. Complete disintegration of the tissue samples by homogenization is crucial to obtain optimal RNA yields. Check for remaining intact tissue particles approximately 2 min after homogenization. If needed, continue to process the sample until completely homogenized.
3. We advise against the use of a sonicator for the lysis of tissue samples, since the considerable heat generated by this device can result in enhanced RNA degradation.
4. This protocol is optimized for rather large and fibrous tissue samples (e.g., interdental gingival papillae). To reliably process considerably smaller samples, we recommend the use of a mortar and pestle to finely pulverize the sample after shock-freezing in liquid nitrogen. The pulverized sample can be resuspended in Trizol reagent and further processed according to our instructions.
5. Samples can/should be stored at -80°C
 - (a) after harvesting (drained tissue sample or homogenized sample in Trizol reagent)
 - (b) after extraction of total RNA (tissue and leukocytes)
 - (c) after IVT reactions
 - (d) after fragmentation
 - (e) to collect a number of samples over time. Simultaneous processing of six to eight samples has proven to be safe and efficient.
6. Instead of using BD Vacutainer CPT tubes already containing Ficoll for cell separation, heparin whole blood can also

be diluted 1:1 with PBS and combined with 3 mL of Ficoll in a 15 mL Falcon tube.

7. Centrifugation without brake is critical for the preparation of peripheral blood monocytic cells (PBMC). Check in advance, as not all standard centrifuges offer this feature.
8. The isolation of monocytes and lymphocytes should be performed from freshly collected blood. If needed, the blood sample can be stored at 4°C for several hours before processing, but the sample should not be frozen. In case of emergency, the cells in the white layer isolated by gradient centrifugation (i.e., peripheral blood monocytic cells) can be frozen in standard cell culture freezing medium (e.g., RPMI [Gibco/Invitrogen] + 10% fetal bovine serum [Gibco] + 10% DMSO [Sigma-Aldrich, St. Louis, MO, USA]) but a significant loss in RNA yield must be expected.
9. When processing the samples, it is highly advisable to wear gloves (and change them frequently) to avoid contaminations with exogenous nucleases. Further, we recommend the use of certified nuclease-free plasticware and filtered tips. Surfaces and instruments should be treated with an RNase removal fluid, e.g., RNase Zap (Ambion). If possible, all RNA-related work should be carried out in dedicated workspace, preferably a hood or a PCR enclosure (e.g., Labconco PCR enclosure, Labconco, Kansas City, MI, USA). The samples should be placed on ice at all times, except when specifically instructed otherwise. Instead of using ice, we found the use of laptop coolers (e.g., Nalgene, Fisher, Rochester, NY, USA) more convenient.
10. Phenol (included in Trizol reagent) is toxic by inhalation, and chloroform is considered a potential carcinogen. Thus, a fume hood, e.g., Safeaire (Fisher Hamilton, Two Rivers, WI, USA) or good ventilation and appropriate personal safety measures (gloves, safety glasses, protective clothing) are imperative when handling these components.
11. The precipitation can also be carried out with isopropanol, resulting in a lower salt content of the pellet. However, we recommend the use of ethanol, since isopropanol pellets are more difficult to see and handle. The salts are subsequently removed by the column-based purification step. Furthermore, the use of round-bottomed 2 mL tubes (instead of 1.5 mL) improves the visibility and handling of the obtained pellet.
12. Pay attention not to lose the pellet when inverting the tube.
13. Do not overdry the pellet, as a completely dried out pellet is transparent and far more difficult to see. It may also fail to dissolve thoroughly in subsequent steps.

14. Caution should be taken to completely dissolve the nucleic acid pellet after the drying step by vigorous pipetting for approximately 1 min/sample.
15. If the A_{260}/A_{280} ratio is not in the range of 1.9–2.1 after total RNA isolation, consider re-cleaning the sample with the Qiagen RNeasy kit.
16. We do not recommend to routinely control the total RNA, cRNA, and fragmented cRNA quality using an Agilent Bioanalyzer or by agarose gel electrophoresis, as these steps are both sample- and time consuming.
17. To increase the RNA concentration in the sample, we recommend to use a vacuum centrifuge (e.g., Vacufuge, Eppendorf, Hamburg, Germany) at 4°C to pellet the nucleic acid and resuspend it in an appropriate volume of RNase-free water.
18. When following the Affymetrix protocols for the in vitro transcription reactions, make sure to fully adhere to their recommendations regarding the temperature for each step (4°C or room temperature). Room temperature should at no point exceed 25°C.
19. We recommend the use of the 10 µL SharpTM (Denville Scientific, Inc.) pipette tips over other products, since these long, slim, marked tips allow for controlled, accurate, and safe pipetting of even miniscule amounts of fluid.

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References

1. Chung, C. H., Bernard, P. S., and Perou, C. M. (2002) Molecular portraits and the family tree of cancer. *Nat. Genet.* **32**(Suppl), 533–540.
2. Quackenbush, J. (2006) Microarray analysis and tumor classification. *N. Engl. J. Med.* **354**, 2463–2472.
3. Hoshida, Y., Villanueva, A., Kobayashi, M., Peix, J., Chiang, D. Y., Camargo, A., Gupta, S., Moore, J., Wrobel, M. J., Lerner, J., Reich, M., Chan, J. A., Glickman, J. N., Ikeda, K., Hashimoto, M., Watanabe, G., Daidone, M. G., Roayaie, S., Schwartz, M., Thung, S., Salvesen, H. B., Gabriel, S.,

- Mazzaferro, V., Bruix, J., Friedman, S. L., Kumada, H., Llovet, J. M., and Golub, T. R. (2008) Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N. Engl. J. Med.* **359**, 1995–2004.
4. Haslett, J. N., and Kunkel, L. M. (2002) Microarray analysis of normal and dystrophic skeletal muscle. *Int. J. Dev. Neurosci.* **20**, 359–365.
 5. Colangelo, V., Schurr, J., Ball, M. J., Pelaez, R. P., Bazan, N. G., and Lukiw, W. J. (2002) Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. *J. Neurosci. Res.* **70**, 462–473.
 6. Haroutunian, V., Katsel, P., and Schmeidler, J. (2007) Transcriptional vulnerability of brain regions in Alzheimer's disease and dementia. *Neurobiol. Aging* **30**, 561–573.
 7. Thornton, S., Sowders, D., Aronow, B., Witte, D. P., Brunner, H. I., Giannini, E. H., and Hirsch, R. (2002) DNA microarray analysis reveals novel gene expression profiles in collagen-induced arthritis. *Clin. Immunol.* **105**, 155–168.
 8. van der Pouw Kraan, T. C., van Baarsen, L. G., Rustenburg, F., Baltus, B., Fero, M., and Verweij, C. L. (2007) Gene expression profiling in rheumatology. *Methods Mol. Med.* **136**, 305–327.
 9. Burke, W. (2003) Genomics as a probe for disease biology. *N. Engl. J. Med.* **349**, 969–974.
 10. Izuhara, K., and Saito, H. (2006) Microarray-based identification of novel biomarkers in asthma. *Allergol. Int.* **55**, 361–367.
 11. Demmer, R. T., Behle, J. H., Wolf, D. L., Handfield, M., Kebschull, M., Celenti, R., Pavlidis, P., and Papapanou, P. N. (2008) Transcriptomes in healthy and diseased gingival tissues. *J. Periodontol.* **79**, 2112–2124.
 12. Papapanou, P. N., Abron, A., Verbitsky, M., Pocolos, D., Yang, J., Qin, J., Fine, J. B., and Pavlidis, P. (2004) Gene expression signatures in chronic and aggressive periodontitis: a pilot study. *Eur. J. Oral Sci.* **112**, 216–223.
 13. Papapanou, P. N., Sedaghatfar, M. H., Demmer, R. T., Wolf, D. L., Yang, J., Roth, G. A., Celenti, R., Belusko, P. B., Lalla, E., and Pavlidis, P. (2007) Periodontal therapy alters gene expression of peripheral blood monocytes. *J. Clin. Periodontol.* **34**, 736–747.

Chapter 25

Bioinformatics Techniques in Microarray Research: Applied Microarray Data Analysis Using R and SAS Software

Ryan T. Demmer, Paul Pavlidis, and Panos N. Papapanou

Abstract

Exploration of the underlying biological mechanisms of disease is useful for many purposes such as the development of novel treatment modalities in addition to informing on-going risk factor research. DNA-microarray technology is a relatively recent and novel approach to conducting genome-wide gene expression studies to identify previously unknown biological pathways associated with disease. The copious data arising from microarray experiments is not conducive to traditional analytical approaches. Beyond the analytical challenges, there are equally important issues related to the interpretation and presentation of results. This chapter outlines appropriate techniques for analyzing microarray data in a fashion that also yields a list of top genes with differential expression in a given experiment. Derivatives of the top genes list can be used as a starting point for the presentation of study results. The list also serves as the basis for additional techniques related to enhanced interpretation and presentation of results. All analyses described in this chapter can be performed using relatively limited computational resources such as a lap top PC with at least 2.0 GB of memory and 2.0 GHz of processing speed.

Key words: Microarray, gene expression, mRNA, bioinformatics, epidemiology, statistics, R, SAS, computational genomics.

1. Introduction

The advent of DNA-microarray technology has created new opportunities for the study of biological pathways relevant to human disease. However, analytical and bioinformatics challenges have developed alongside the advances in basic sciences accomplished by the use of genomic approaches. From an analytical standpoint, two of the greatest challenges are to (i) efficiently identify relevant biological patterns related to the disease process

and (ii) handle the statistical issue of multiple comparisons. In this chapter, we provide an overview to help initiate researchers who are unfamiliar with microarray data analysis methods and concepts. Our aim is to give explicit, step-by-step examples of how one may conduct an analysis of their own data. It is expected that the reader will have some basic knowledge in statistics, causal inference, and data analysis using the relevant statistical analysis packages described herein. The analytical examples presented in this chapter are based on experiments using single channel Affymetrix GeneChips[®] but most of the analytical strategies and approaches discussed are applicable to other microarray designs as well.

1.1. Broad Overview of Microarray Data Analysis Concepts

Analytical approaches in microarray research generally fall into one of two broad categories defined as either supervised or unsupervised analysis. It is common to use both approaches when analyzing results from a single experiment but the necessary analytical approach will vary according to the design and aims of each experiment. A brief description of unsupervised and supervised analyses is provided below followed by a description of the experimental design on which we will focus our analytical methods in this chapter. For a more comprehensive overview and discussion of microarray data analysis, please *see* Quackenbush (1).

1.1.1. Unsupervised Analysis (Class Discovery)

Genomic research has contributed significantly to the identification of novel biological pathways underlying human disease. This has been accomplished partly through the use of unsupervised methods that can identify novel gene expression patterns. These techniques ignore any a priori information about the samples such as diagnosis, disease state, histologic characteristics but rather seek to identify potentially biologically meaningful patterns of expression. Various forms of cluster analysis, such as hierarchical cluster analysis or k-means clustering, have been utilized for these purposes. The premise of cluster analysis is to assign a measure of similarity to gene expression profiles. Expression similarity can be observed (i) between genes (across samples); or (ii) between samples (across genes); or (iii) as a combination of i and ii. A detailed description of cluster analysis theory and application is beyond the scope of this chapter and the specific analytical methods we describe will focus on a specific application of supervised analysis.

1.1.2. Supervised Analysis (Differential Expression)

In supervised analyses biological samples or participants are classified a priori based on relevant characteristics (i.e., disease diagnosis). These classifications then serve as independent variables in statistical models predicting variation in gene expression level (the dependent variable). The null hypothesis for each gene is that expression will not vary across the predefined classes. There are various approaches to supervised analysis and in this chapter,

we will focus on appropriate techniques for conducting a supervised analysis for the identification of genes that are differentially expressed in states of periodontal health vs. disease. Therefore, our example will identify “top genes” with biological relevance to periodontal disease. We will use two examples stemming from the same study design but using two different statistical methods. A brief description of the study design and scientific aim follows. We have recently studied gene expression signatures in healthy and diseased gingival tissues (2) in 90 non-smokers, 63 with chronic and 27 with aggressive periodontitis, each contributing with ≥ 2 “diseased” interproximal papillae (with bleeding on probing, probing pocket depth ≥ 4 mm, and clinical attachment loss ≥ 3 mm) and a “healthy” papilla. This design allowed us to test whether expression levels of various genes are different when comparing diseased vs. healthy gingival tissue. The steps for identifying a list of differentially expressed genes using either mixed effects statistical models or two-sample t tests are outlined below.

All analyses described in this chapter were tested using both: i) a laptop PC running Windows Vista, with a 2.8 GHz processor and 3.0 GB of RAM; and ii) a laptop MacBook Pro, with a 2.53 GHz processor and 4.0 GB of RAM. We expect that the code will also work on other platforms such as UNIX, LINUX & XP but minor adjustments might be required for these systems.

2. Materials

2.1. Data

This chapter will assume that all original data files are located on the C drive in a folder named microarray: “C:\microarray”

1. Affymetrix CEL files for each experimental sample (*see Note 1*).
2. Gene annotation file (*see Note 2*).
3. Experimental design data file (*see Note 3*).

2.2. Statistical Analysis Software

1. R software and related analysis packages (*see Note 4*).
2. SAS software (optional, *see Note 5*).

3. Methods

In this section we provide explicit step-by-step instructions for manipulating and analyzing gene expression data. The analysis is based on our previously published work (2) as described above. It should be noted at the outset that there are no “absolutes”

when it comes to developing an analytical strategy and corresponding computer code for analyzing gene expression data. The examples we provide are designed to be readable, transparent, and functional. However, in many situations they are not necessarily optimized for efficiency or programming eloquence. To accomplish efficiency of this nature, an understanding of computing, bioinformatics, and statistics beyond the scope of this chapter is required.

*Also, please note that downloadable text files containing the R and SAS code presented in this chapter are available at:
<http://www.chibi.ubc.ca/faculty/pavlidis/demmer-methods>

3.1. Create List of Genes, p-Values and q-Values Using R

Open R on your PC and paste the following commands to the command line. The prompt symbols (“>”) should not be entered by the user; prompt symbols will appear by default in the R program and are presently included to reflect the beginning of a new command that should be typed directly into the R command line. Comments are denoted with a pound sign (#) at the beginning of the comment. Comments should not be pasted to the R command line. Finally, be aware that R code is case sensitive.

3.1.1. Input Data Files and Normalize Expression Data

1. #Set working directory in R (*see Note 6*).
 > setwd("C:/microarray")
2. #Load the affy library (*see Note 4*)
 > library(affy)
3. #Create normalized expression data based on all CEL files in the working directory
 > e<-justRMA(destructive=TRUE)
4. #Write the expression data (*see Note 7*) in R object "e" to a text file in the working directory
 > write.exprs(e, file="expressionData.txt")
5. #Read EDDF into an R object.
 > design<-read.delim("EDDF.txt", row.names=1, header=T)
6. #Remove .CEL file extensions and X "characters" from expression data column names (*see Note 8*)
 > d<-read.table("expressionData.txt", header=T, row.names=1)
 > names(d)<-gsub(".CEL", "", gsub("X", "", names(d)))
 > d<-round(d, 5)
7. #Write a permanent expression data file to the working directory
 > write.table(d, file="expressionDataFinal.txt", quote=F, sep='\t')

3.1.2. Modify Data

1. #Read the expressionDataFinal into R as an object
`>expression<-read.delim("expressionDataFinal.txt",
row.names=1, header=T)`
2. #Remove "X" characters from column names
(corresponding to Sample_ID numbers).
"X" characters are inserted automatically by the
read.delim function
`>names(expression) <-sapply(strsplit(names
(expression), "X"),
function(x) {x[2]}))`
3. #Arrange expression data in ascending ID order
(arranges columns from left to right)
`>expression<-expression [,as.character(sort
(as.numeric(names(expression))))]`
4. #Arrange experimental design data file in
ascending ID order (arranges rows from top to
bottom)
`>designOrder<-order(as.numeric(row.names(design)))
>design<-design[designOrder,]`
5. #Reduce the design and expression R objects to
include only biological samples that appear in
both objects.
`>exprsID<-data.frame(names(expression))
>designID<-data.frame(row.names(design))
>names(exprsID)<-c("ID")
>names(designID)<-c("ID")
>idoverlap<-merge(exprsID, designID, by = "ID")
>row.names(idoverlap)<-idoverlap$ID
>design<-design[row.names(idoverlap),]
>expression<-expression[,row.names(idoverlap)]`

3.1.3. Run Linear Mixed Effects Statistical Models

The following steps will run a linear mixed effects ANOVA (3) analysis exploring whether expression levels for each probe set (dependent variable) are differential by level of the biological sample status (independent variable – modeled as healthy vs. diseased gingival tissue in this example). We first set up a function in R and apply the function to the actual data and extract p -values from each regression. The end result (for this specific experiment) will be a list of 54,675 p -values – one p -value for each probe set on the particular Affymetrix GeneChip used in our experiments (Human Genome U133 Plus 2.0 Array).

1. #Load the "nlme" library
`>library(nlme)`
2. #Create a function that will run the mixed model
regression for all 54,675 probe sets on our
GeneChip
`>mm.funHD<-function(x)`


```

{
d<-data.frame(x, design[,c("Patient",
"Diseased_Tissue")])
names(d)<-c("E", "Patient", "Diseased")
r<-NULL
try(silent=T, r<-anova(lme(E ~ Diseased,
random = ~ 1 | Patient, data=d))[,4][2])
r
}

```

3. #Apply the function to the expression data and save the result to an R object titled "hlthDis"

```
>hlthDis<-apply(expression, 1, mm.funHD)
```
4. #Step #3 will take time (anywhere from seconds to many minutes depending on the memory and processor speed of the computer being used. Therefore it is advisable to save the R image so that the analysis does not need to be rerun in the future

```
>save.image()
```
5. #Replace "NULL" values with "NA" because R understands that NA = missing data (missing p-value in this example) whereas R does not know how to handle "NULL"

```
>hlthDis2<-gsub("NULL","NA",hlthDis)
>hlthDis2<-as.numeric(hlthDis2)
>names(hlthDis2)<- names(hlthDis)
```
6. #Creates a text file in the working directory containing all probe sets and their corresponding p-values in ascending p-value order

```
>write.table(sort(hlthDis2,na.last=TRUE),
file="hlthDis.rem.txt",col.names=
"probe p-value", quote=F, sep= '\t')
```
7. Create a reduced text file in the working directory that removes any probe sets with p-values = "NA" because these values can prevent the q-value function from working in subsequent steps. If there are no "NA" values in "hlthDis.rem.txt" then this step is unnecessary.

```
>hlthDis3<-hlthDis2[which(hlthDis2 != "NA")]
>write.table(sort(hlthDis3,na.last=TRUE),file=
"hlthDisNoNA.rem.txt",col.names="probe p-value",
quote=F, sep='\t')
```

3.1.4. Obtain q-Values and Merge q-Values with p-Values (see Note 9)

1. #Load qvalue library

```
>library(qvalue)
```
2. #Read the list of p-values created in **Section 3.1.3** into an R object

```

>pvalues<-read.table("hlthDisNoNA.rem.txt",
  skip=1,row.names=1)
>pvalues2<-pvalues[,1]
3. #Apply the qvalue function to generate a table
   indicating the number of significant calls for
   both the raw p-values and for the calculated
   q-values using a set of cutoffs given by "cuts"
   (see Notes 9,10).
>summary(qvalue(pvalues2), cuts = c(9.14e-07,
  0.001, 0.01, 0.025, 0.05, 0.1, 1))
4. #Create a vector of the q-values and merge with
   p-values
>q<-qvalue(pvalues2)$qvalues
>q<-data.frame(q)
>qp<-data.frame(row.names=row.names(pvalues),
  q, pvalues)
>names(qp)<-c("qvalue","pvalue")
>porder<-order(qp[, "pvalue"])
>qp<-qp[porder,]
>qpfinal<-data.frame(row.names(qp),qp$qvalue,
  qp$pvalue)
>names(qpfinal)<-c("probe","qvalue","pvalue")
>write.table(qpfinal,file="hlthDis.qp.rem.txt",
  row.names=F, quote=F, sep='\t')

```

3.1.5. Merge *p*-Values and *q*-Values with Gene Annotation File

At this point, we have generated a list of *p*-values and *q*-values corresponding to every probe set on the microarray chip (in addition to a summary corresponding to the number of significant calls using both *q*-value and *p*-value significance criteria). Now, in a final step, merge gene annotations to the file containing *p*-values and *q*-values (“hlthDis.qp.rem.txt” created in **Section 3.1.4**) and reduce the data to a file containing a list of “top genes.”

```

1. #Read the gene annotation file into an R object
   (see Note 11)
>annotations<-read.delim("C:/microarray/HG-U133_
  Plus_2_bioproc.an.txt", col.names=
  c("probe", "gene", "description",
  "G0terms"), sep="\t", fill=T)
>annotations<-data.frame(annotations)
>probeorderannot<-order(annotations[, "Probe"])
2. #Order the annotation file by probe ID
>annotations2<-annotations[probeorderannot,]
3. #Order "qpfinal" by probe ID
>probeorder<-order(qpfinal[, "probe"])
>qpfinal<-qpfinal[probeorder,]
4. #Combine the modified R objects containing the
   gene annotations ("annotations2") and the
   p-values and q-values ("qpfinal")

```

```

>final<-merge(qpfinal, annotations2, by="probe")
#(see Note 12)
>FinalPvalueOrder<-final[order(final[, "pvalue"]),]
>FinalPvalueOrder<-FinalPvalueOrder[, c("probe",
    "gene", "description", "pvalue", "qvalue")]
>write.table(FinalPvalueOrder, file=
    "FinalPvalueOrder.txt", row.names=F, quote=F,
    sep='\t')
5. #Alternatively, if you only want a table
    including genes with a qvalue < 0.05 then do:
>FinalPvalueOrder<-FinalPvalueOrder
    [which(FinalPvalueOrder[, "qvalue"] < 0.05),]
>write.table(FinalPvalueOrder, file=
    "FinalPvalueOrder.txt", row.names=F, quote=F,
    sep='\t')

```

3.2. Two Sample TTEST in R

The study design we have based our analytical approach on thus far is typical of clinical research in humans. Because of the repeated measures occurring within person, a sophisticated statistical analysis method was required (Mixed Model Regressions). However, other designs (and subsequent hypothesis testing scenarios) are also common in clinical research. For example, consider the following scientific question currently under investigation (4): “Does gene expression in diseased gingival tissue differ between patients diagnosed with chronic vs. aggressive periodontitis?” Now assume for the moment that the experimental design collects one diseased gingival tissue sample per patient, among $n=10$ patients ($n=5$ with chronic periodontitis and $n=5$ with aggressive periodontitis). The gene expression data obtained from this experiment are conducive to a classical two-sample t test which compares mean gingival tissue gene expression between the two groups of five patients. The following code demonstrates the appropriate steps to generate a list of top genes differentially expressed between these two hypothetical patient groups (see **Note 13**).

3.2.1. Modify Data

```

1. #To calculate two-sample t-tests in the fashion
    described above, we first need to modify the
    previously created R data objects, "design" and
    "expression" by restricting the data to the
    first diseased sample per patient.
>ttestobs<-which(design$Sample_Number == 1
    & design$Diseased_Tissue == 1)
>designT<-design[ttestobs,]
2. #Restrict expression to include only samples in
    the modified design, "designT"
expressionT<-expression[, ttestobs]

```

3. #Create a function that will run two-sample t-tests for all 54,675 probe sets on the GeneChip.

```
>ttest.fundiag<-function(x)
{
  designT2<-data.frame(x, designT[, "Diagnosis"])
  names(designT2)<-c("E", "Diagnosis")
  r<-NULL
  try(silent=T, r<-t.test(E~Diagnosis,
    data = designT2, var.equal=T)["p.value"])
  r
}
```
4. #Apply the function and write the results to the user defined R object "DiagnosisT".

```
>DiagnosisT<-apply(expressionT, 1, ttest.fundiag)
```
5. #The next series of data manipulations allow for future sorting by p-value and saving results to a text file

```
>DiagnosisT2<-unlist(DiagnosisT)
>DiagnosisT2<-gsub("NULL", "NA", DiagnosisT2)
>DiagnosisT2<-as.numeric(DiagnosisT2)
>names(DiagnosisT2)<- names(DiagnosisT)
```
6. #Creates a text file in the working directory containing all probe sets and their corresponding two-sample t-test p-values in ascending p-value order

```
>write.table(sort(DiagnosisT2, na.last=TRUE),
  file="Final_t-test_PvalueOrder.txt",
  row.names=T, quote=F, col.names="probe pvalue",
  sep='\t')
```
7. #At this point you can now use the exact same methods in **Sections 3.1.4** and **3.1.5** to create a final text file containing genes, descriptions, p-values and q-values. Simply substitute the old p-value list "hlthDisNoNA.Rem.txt" with your new p-value list "Final_t-test_PvalueOrder.txt".

3.3. Create List of Genes, p-Values and q-Values Using SAS

The following sections generate results identical to those in **Sections 3.1** and **3.2**; only now we are using SAS software (*see Note 5*) to perform the analyses. The SAS examples also include the necessary code to generate gene expression fold changes (*see Note 14*). In SAS examples, a forward slash asterisk (/*) note the beginning of a comment and an asterisk forward slash (*/) note the end of a comment. We have also used ALL CAPS for SAS keywords and mixed case for user-supplied text in keeping with typographical conventions used in other SAS textbooks (5).

3.3.1. *Import Data Files*

1. */*Import Gene Annotation File*/*
DATA WORK.annotations;
INFILE "C:\microarray\GeneAnnotationFile.txt"
DELIMITER='09'x
MISSOVER DSD LRECL=32767 FIRSTOBS=2;
INFORMAT probe \$27.;
INFORMAT Gene \$10.;
INFORMAT Description \$100.;
INFORMAT GOTerms \$12.;
FORMAT probe \$27.;
FORMAT Gene \$10.;
FORMAT Description \$100.;
FORMAT GOTerms \$12.;
INPUT probe \$ Gene \$ Description \$ GOTerms \$;
RUN;
2. */*Sort Gene Annotation File by probe to allow for merging in later data steps*/*
PROC SORT DATA = annotations;
BY probe;
RUN;
3. */*Import Experimental Design Data File EDDF.txt*/*
DATA WORK.design;
INFILE "C:\microarray\EDDF.txt" DELIMITER='09'x
MISSOVER DSD LRECL=32767 FIRSTOBS=2 ;
INFORMAT Sample_ID 8.1;
INFORMAT Patient 8.;
INFORMAT Sample_Number 1.;
INFORMAT Diseased_Tissue 1.;
INFORMAT Diagnosis 1.;

FORMAT Sample_ID 8.1;
FORMAT Patient 8.;
FORMAT Sample_Number 1.;
FORMAT Diseased_Tissue 1.;
FORMAT Diagnosis 1.;

INPUT Sample_ID Patient Sample_Number
Diseased_Tissue Diagnosis;
RUN;
4. */*Import Normalized Expression Data (see Note 15).*
When importing the normalize gene expression data, it will be necessary for the user to ensure that the order of INFORMAT, FORMAT and INPUT statements below, are identical to the column ordering (which correspond to each tissue sample) in the "expressionData.txt" generated in **Section 3.1.1**. If the orders do not match, the wrong expression data will be matched to each tissue sample (*see Note 16*).*/
DATA WORK.EXPRIDORDER;
%LET _EFIERR_ = 0;

```

INFILE 'C:\microarray\expressionDataFinal.txt'
delimiter='09'x
MISSOVER DSD LRECL=32767 FIRSTOBS=2 ;
INFORMAT probe $27.;
INFORMAT ID_1_1 BEST32.;
INFORMAT ID_1_2 BEST32.;
INFORMAT ID_1_4 BEST32.;
INFORMAT ID_2_1 BEST32.;
INFORMAT ID_2_2 BEST32.;
INFORMAT ID_2_4 BEST32.;
INFORMAT ID_4_3 BEST32.;
FORMAT probe $27.;
FORMAT ID_1_1 BEST12.;
FORMAT ID_1_2 BEST12.;
FORMAT ID_1_4 BEST12.;
FORMAT ID_2_1 BEST12.;
FORMAT ID_2_2 BEST12.;
FORMAT ID_2_4 BEST12.;
FORMAT ID_4_3 BEST12.;
INPUT probe $ ID_1_1 ID_1_2 ID_1_4 ID_2_1 ID_2_2
        ID_2_4 ID_4_3;
IF _ERROR_ THEN CALL SYMPUTX ('_EFIERR_',1);
RUN;

```

3.3.2. Manipulate Gene Expression Data Set in SAS

1. /*Convert the data structure from "wide" to "long" form (see **Note 17**)*/

```

DATA expr2;
SET expridorder;
ARRAY origida [*] ID_1_1--ID_4_3;
ARRAY newida [7]$ _TEMPORARY_;
DO i = 1 TO DIM(origida);
newida[i] = VNAME(origida[i]);
END;
DO i = 1 to DIM(origida);
exprs=origida[i]; id=newida[i]; OUTPUT;
END;
KEEP probe exprs id;
RUN;

```
2. /*Translate id number into same format as the format used the design data set. This allows the two data sets to be merged in future data steps*/

```

DATA expr2;
SET expr2;
pat=SCAN(id,2,'_');
pat2=SCAN(id,3,'_');
id=TRIM(pat)||"_"||TRIM(pat2);
id = TRANWRD(id,'_','.');
id2 = INPUT(id,5.);

```

```

        KEEP probe exprs id2;
    RUN;

3. /*Sort expression and design data sets by sample
   id number so they can be merged*/
PROC SORT DATA=design; BY Sample_id; RUN;
PROC SORT DATA=expr2; BY id2; RUN;

4. /*Merge expression data with design data by sample
   id number*/
DATA expr3;
MERGE expr2 (RENAME=(id2=id) IN=inexpr) design
  (RENAME=(Sample_ID=id)IN=indesign);
BY id;
IF inexpr and indesign;
RUN;

```

3.3.3. Mixed Model Regressions

```

1. /*Sort data set by probe to allow regressions to
   be run by probe*/
PROC SORT DATA=expr3;
BY probe;
RUN;

2. /*see Note 18 for further information on SAS
   system options used and the output delivery
   system in SAS*/
OPTIONS MSGLEVEL=N NONOTES STIMER;

3. /*Mixed model regressions*/
ODS LISTING CLOSE;
ODS RESULTS OFF;
PROC MIXED DATA = expr3;
BY probe;
ODS OUTPUT TESTS3=perio (KEEP = probe Probf);
  *keeping p-values from F-statistic;
MODEL exprs = Diseased_Tissue/S;
RANDOM INTERCEPT/ SUBJECT = patient;
RUN;QUIT;
ODS RESULTS ON;
ODS LISTING;
OPTIONS MSGLEVEL=I NOTES;

```

3.3.4. Merge SAS Intermediate Data Sets, Create Gene Expression Fold Changes and q-Values

```

1. /*For each probe, create mean expression values
   across patient and within Diseased_Tissue status
   (Diseased (1) vs. Healthy (0)). This will allow
   for the creation of fold changes in a future
   data step*/
PROC MEANS DATA = expr3 NOPRINT NWAY;
CLASS probe Diseased_Tissue;
VAR exprs;
OUTPUT OUT = means (DROP = _TYPE_ _FREQ_)

```

```

        MEAN = exprs;
    RUN;
2. /*Get mean expression values for healthy and
   diseased tissue into one observation*/
DATA means1;
SET means;
BY probe;
IF FIRST.probe THEN DO;
IF Diseased_Tissue = 0 THEN exprs0 = exprs;
RETAIN exprs0;
END;
IF Diseased_Tissue = 1 THEN exprs1 = exprs;
IF LAST.probe THEN OUTPUT;
RUN;

DATA final;
MERGE annotations means1 perio
  (RENAME=(probf=pvalue) IN=inperio);
BY probe; IF inperio;
FORMAT pvalue e16.;
RUN;
PROC SORT DATA = final;
BY pvalue;
RUN;
DATA final;
SET final;
obsnum+1;
/*(see Note 9)*/
qvalue = (pvalue*54675)/obsnum;
FC = 2** (exprs1-exprs0);
/*Calculate the absolute fold change so up- and
down-regulated genes can be compared on the
same scale*/
absoluteFC = 2** (ABS(exprs1-exprs0));
KEEP Gene Description probe pvalue qvalue
      absoluteFC FC;
RUN;
3. /*Sort the final data set by absolutFC -
   see Note 14 */
PROC SORT DATA = final; (see Note 19)
BY DESCENDING absoluteFC pvalue;
RUN;

```

3.3.5. Create Final Excel Spreadsheet

```

1. /*Create a final Excel spreadsheet containing the
   results for all genes sorted by absolute fold
   change*/
ODS LISTING CLOSE;
ODS HTML BODY = "C:\microarray\TopGenes.xls"
  style=minimal;
PROC PRINT DATA = final NOOBS;
RUN;

```



```
ODS HTML CLOSE;
ODS LISTING;
```

3.4. Two Sample TTEST in SAS

Refer to [Section 3.2](#) and [Note 13](#) for a brief introduction to the scientific question being addressed in the following SAS code.

1. /*Restrict the data set "expr3" created in [Section 3.3.2](#) step 4, to include the appropriate samples (*see Note 13*)*/

```
DATA expr3;
SET expr3;
WHERE Diseased_Tissue = 1 and Sample_Number = 1;
KEEP id probe exprs Diagnosis;
RUN;
```
2. /*The data set expr3 should already be sorted by probe but redo to be sure*/

```
PROC SORT DATA = expr3;
BY probe;
RUN;
```
3. /*Run t-tests for all 54,675 probe sets on the microarray chip*/

```
ODS LISTING CLOSE;
ODS RESULTS OFF;
PROC TTEST DATA = expr3;
BY probe;
CLASS Diagnosis;
VAR exprs;
ODS OUTPUT Statistics=stats
  (KEEP = probe class mean) Ttests=ttests
  (KEEP = probe variances probt);
RUN;
ODS RESULTS ON;
ODS LISTING;
```
4. /*Modify the data sets "ttests" and "stats" created in the ODS OUTPUT statement from step 3*/

```
DATA ttests; SET ttests (RENAME=(probt=pvalue));
WHERE variances = "Equal";
KEEP probe pvalue;
run;
DATA stats;
SET stats;
WHERE class = "Diff (1-2)";
KEEP probe mean;
RUN;
```
5. /*Sort SAS data sets for merging by probe*/

```
PROC SORT DATA = stats;
BY probe;
RUN;
PROC SORT DATA = ttests;
```

```

BY probe;
RUN;
PROC SORT DATA = annotations;
BY probe;
RUN;
6. /*Merge necessary SAS data sets, create q-values
   and fold changes*/
DATA final;
MERGE annotations ttests stats;
BY probe;
FORMAT pvalue e16.;
RUN;
PROC SORT DATA = final;
BY pvalue;
RUN;
DATA final;
SET final;
obsnum+1;
qvalue = (pvalue*54675)/obsnum;
FC = 2** (mean); /*Chronic vs. Aggressive*/
absoluteFC = 2** (ABS(mean));
KEEP probe Gene Description pvalue qvalue fc
      absoluteFC;
RUN;
7. /*Sort the final data set by absolutFC -
   see Note 14*/
PROC SORT DATA = final;
BY DESCENDING absoluteFC;
RUN;
8. /*Create final Excel spreadsheet*/
ODS LISTING CLOSE; /*prevents printing to
   output screen;
ODS HTML BODY = "C:\microarray\TopGenesTTEST.xls"
   STYLE=minimal;
PROC PRINT DATA = final NOOBS; RUN;
ODS HTML CLOSE;
ODS LISTING;

```

3.5. Gene Ontology Analysis

After performing the appropriate statistical analysis to determine a level of statistical significance for each gene, it is often useful to identify groups of affected genes with similar biological function. Gene ontology analysis is an emerging method for this goal of grouping genes. Step-by-step instructions for a gene ontology analysis are beyond the scope of this chapter. However, two high quality and readily available tools are available for free download online. The user's manuals of these programs are sufficient for novice users to conduct a gene ontology analysis using the *p*-value

list(s) generated above. We suggest the following two programs and provide their respective World Wide Web addresses, where more information can be found:

ErmineJ (6): <http://www.bioinformatics.ubc.ca/ermineJ/index.html>

Pathway Express (7, 8): <http://vortex.cs.wayne.edu/Projects.html>

4. Notes

1. Affymetrix CEL files are created by Affymetrix image analysis software. The CEL file stores the results of the intensity calculations for each probe on the GeneChip. The intensity is based on the pixel values of the DAT file. This information is used to generate an expression level for each probe and thereby each gene on the GeneChip. There is one CEL file for each biological sample collected.
2. Gene annotations files can be downloaded directly from the Affymetrix web site or alternatively, custom files developed by other research groups are also available for free download from the internet. In our studies, we have used the annotation file developed by Dr. Paul Pavlidis and colleagues (University of British Columbia, Canada). A detailed description of the annotation files can be found at the following WWW address: <http://www.bioinformatics.ubc.ca/microannots/>

We recommend using the biological processes only version of the annotations corresponding to the microarray chip in your experiment. For the current example, the appropriate annotations file can be downloaded directly at the following WWW address: http://www.bioinformatics.ubc.ca/microannots/HG-U133_Plus_2_bioproc.an.zip

After downloading this zipped file, you will need to unzip and save the file as a tab delimited text file in your working directory. If the file is not saved as a tab delimited text file, it will not import properly (this is true for both R and SAS imports). **Table 25.1** provides a truncated example of a typical gene annotation file structure.

3. The Experimental Design Data File (EDDF) contains experimental design information that will be used to merge characteristics of each sample in the experiment (i.e., sample ID, which samples are healthy or diseased; treated or untreated) and merge this information with the

Table 25.1
Truncated example of a gene annotation file

Probe ID	Gene	Description	GOTerms
91580_at	LRTM1	Leucine-rich repeats and transmembrane domains 1	
90610_at	LRCH4	Leucine-rich repeats and calponin homology (CH) domain containing 4	GO:0007399...
90265_at	CENTA1	Centaurin, alpha 1	GO:0050789...
89977_at	FLJ20581	Hypothetical protein FLJ20581	GO:0007582...
89948_at	C20orf67	Chromosome 20 open reading frame 67	
89476_r_at	NPEPL1	Aminopeptidase-like 1	GO:0044237...
87100_at	ABHD2	Abhydrolase domain containing 2	GO:0008150
823_at	CX3CL1	Chemokine (C-X3-C motif) ligand 1	GO:0009605

An example of eight probe sets (out of 54,675 total) and their descriptions included on the Affymetrix HG-U133 GeneChip.

Table 25.2
Truncated example of an experimental design data file

Sample_ID	Patient	Sample_Number	Diseased_Tissue	Diagnosis
1.1	1	1	1	1
1.2	1	2	1	1
1.3	1	3	0	1
2.1	2	1	1	2
2.2	2	2	1	2
2.3	2	3	0	2
3.1	3	1	1	2
3.2	3	2	1	2
3.3	3	3	0	2
4.1	4	1	1	1
4.2	4	2	1	1
4.3	4	3	0	1

Variable key: “Diseased_Tissue”, 1 = diseased; 0 = healthy. “Diagnosis”, 1 = Chronic; 2 = Aggressive.

corresponding expression data. **Table 25.2** provides a truncated example of the EDDF structure.

4. R software is freely available. Visit the following web site for information on the product and instructions regarding free download: <http://cran.r-project.org/>

In addition to the base R software, download the following packages from the CRAN web site: “nlme”, “qvalue”.

Also download and install Bioconductor, offered by the Bioconductor Project: <http://www.bioconductor.org/packages/release/bioc/> In addition the “affy” package, will need to be downloaded and installed. Additional Bioconductor packages will likely be required (such as “Biobase”) depending on the user’s current R setup. Follow the prompts given by R when attempting to install the “affy” package.

5. SAS is a widely used data management and statistical analysis software package. SAS is not required to complete the analyses described in **Sections 3.1** or **3.2**. The SAS examples provided in **Sections 3.3** and **3.4** generate (almost) identical results to those provided in R and we include sections based on SAS simply because this software is so widely used. Users without any prior SAS experience are advised to use the freely available R software only.
6. Working directory: R for PC recognizes forward slashes (/) in the file path. SAS recognizes back slashes (\).
7. The data structure of most microarray experiments is different than traditional experiments which have a limited number of study outcomes. **Table 25.2** is an example of a traditional data structure in which study participants (or biological samples) are presented in rows and study outcomes or patient characteristics such as blood biomarkers or diagnosis are presented in columns. This type of table is commonly created by an investigator using readily available database programs such as Microsoft Access or Excel. However, in the context of microarray research, a data structure that can more efficiently handle large amounts of data is generally required. **Table 25.3** presents a typical microarray data structure where participants (or biological samples) are presented in columns while gene expression levels for the various genes under study are presented in rows. The initial gene expression data files created in **Section 3.1.1** will follow the format presented in **Table 25.3**.
8. This series of commands will remove “X” characters and .CEL file extensions from the variable names (column names) in the normalized expression file created in **Section 3.1.1**, step 4.

Removing the “X” character is specific to the variable naming convention used in this chapter. As seen in **Table 25.3**, our variable names (which correspond to tissue samples) are numeric and not character. Because R does not handle numeric variable names, an “X” is automatically added to the variable name by R to avoid this conflict. Consequently, we need to remove the “X” so that the variable

Table 25.3
Truncated example of a gene expression data matrix

Probe	1.1	1.2	1.3	2.1
1007_s_at	10.14741	10.46277	10.43202	9.71754
1053_at	6.52359	6.77471	7.05892	6.6885
117_at	6.98609	6.92772	6.60945	8.07533
121_at	8.16319	8.02055	8.35759	8.44039
1255_g_at	3.27397	3.27663	3.32964	3.43425
1294_at	7.51381	7.28304	7.06534	7.32475
1316_at	5.14637	5.27665	5.14162	4.93963

names in **Table 25.3** match the Sample_IDs in **Table 25.2**. Accordingly, the CEL file extensions need to be removed for the same reason.

The “round” function is also introduced. The “round” function, rounds numerical values to a specified number of digits. This step is performed to reduce file size.

- To paraphrase Storey & Tibshirani (9), the q -value provides a measure of each probe set’s significance, automatically taking into account the fact that thousands of hypotheses are simultaneously being tested (i.e., in the current example, the expression of 54,675 probe sets is being compared between healthy and diseased gingival tissue). The q -value corresponds directly to the false discovery rate (FDR) and the FDR in turn refers to the percentage of all “significant” statistical tests that are truly null.

Results from the q value function will appear similar to those shown in **Table 25.4**. The interpretation based on **Table 25.4** is that 39,690 probe sets were identified with a false discovery rate of <0.05 and 12,743 probe sets were identified with a p -value $<9.14 \times 10^{-7}$.

Table 25.4
Example of “qvalue” function results

Cumulative number of significant calls

	<9.14e-07	<0.001	<0.01	<0.025	<0.05	<0.1	<1
p -value	12,743	23,036	29,024	32,005	34,625	37,622	54,675
q -value	12,698	24,405	31,865	35,848	39,690	44,656	54,675

10. We use 9.14×10^{-7} as the lowest cutoff in this example because this corresponds to the very conservative Bonferroni adjustment (calculated as $p\text{-value}/\text{number of statistical tests}$, or $0.05/54,675$ in the current example). A gene chip with a different number of probe sets would use a different Bonferroni adjustment.
11. The `col.names` option provides names for the variables in the raw annotation file. This allows a different name to be assigned to the variables in the original data file.
12. After this step you could print to the screen, a subset of the R object “final”. The object “final” contains the probe variable twice and printing allows for visual confirmation that the correct annotations were merged with the correct p -values and q -values. The following code in R will print 15 rows and all columns of the R object: `<final[1:15,]`
13. In this example, we should clarify that (i) the gingival tissue samples arise from the same patient population as in the earlier “healthy vs. diseased” example, (ii) only the first diseased sample collected from each patient is used in this analysis. This is done because we wish to demonstrate an approach using two-sample t -test – a common statistical methods used in clinical research, and (iii) mixed model approaches could also be applied in this setting.

It should also be noted that although this example uses a study design applied to human subjects, the basic scientist could use the same techniques. For example, instead of patients the design might use mice as the experimental unit and rather than observing a diagnosis, the “exposure” of interest might be treatment with an experimental drug in a test group of mice vs. treatment with saline in a control group.

14. Fold change refers to the relative difference in gene expression between two groups of samples. For example, if we find that the mean expression level is 10,000 among diseased gingival tissues and 1,000 among healthy gingival tissues, the fold change for diseased vs. healthy tissue is 10.
15. The authors are currently unaware of any functions included in Base SAS that will convert raw data from .CEL files to a normalized SAS data set. Therefore, in this example, the user will still be required to use R to get normalized expression data (*see* **Section 3.1.1**). To conserve space we have only provided a truncated version of Expression Data import code. The `INFORMAT`, `FORMAT`, and `INPUT` statements should have a statement for each tissue sample in the experiment.

16. The import code we have supplied ignores the variable names supplied in the original “expressionData.txt” file. The `FIRSTOBS = 2` option tells SAS to begin reading data at line 2 on the input file and the variable names are explicitly provided in the `INFORMAT`, `FORMAT`, and `INPUT` statements. There is no direct link between the variable names the user provides and the column names of the input file (expressionData.txt). Consequently, it is imperative that the variable names in the `INFORMAT`, `FORMAT`, and `INPUT` statements are ordered *identically* to the columns (which correspond to each tissue sample) in the “expressionData.txt” input file generated in **Section 3.1.1**. If the orders do not match, the wrong expression data will be matched to each tissue sample id.

The variable naming convention (names for each column) used for tissue samples in this example (ID_1_1, ID_1_2, etc.) combines the patient and sample ID such that ID_1_1 refers to patient 1, tissue sample 1. We begin the variable name with ID and use underscores as delimiters because SAS will not accept variable names that begin with numbers. Similarly, SAS will not accept variable names that contain ‘.’ symbols. Therefore, the user is free to modify the naming convention in a manner that suits their data or preferences best but it will necessitate subsequent revisions throughout the remaining SAS code.

17. The file “expressionDataFinal.txt” is arranged as demonstrated in **Table 25.3**. The steps in this section convert the data structure to conform to a format outlined in **Table 25.5**, in which expression data are shown in the long form for one patient, two samples, three probes. This is necessary to accommodate the SAS statistical analysis procedures in subsequent steps.

Table 25.5
Truncated example gene expression data matrix converted from wide to long form

Probe	Exprs	Sample_ID	Patient	Sample_Number
1007_s_at	10.14741	1.1	1	1
1053_at	6.52359	1.1	1	1
117_at	6.98609	1.1	1	1
1007_s_at	10.46277	1.2	1	2
1053_at	6.77471	1.2	1	2
117_at	6.92772	1.2	1	2

18. Because the number of analyses (one for each probe set on the microarray) is generally very large, it is necessary to suppress normal printing to the SAS log and output windows. If the printing is not suppressed, most machines will run out of memory and the analysis will fail. By using SAS system options and ODS commands, this output can be easily suppressed. A brief overview of the options used presently is given below.

NOTES | NONOTES – controls whether notes (messages beginning with NOTE) are written to the SAS log. However, NONOTES does not suppress error or warning messages.

MSGLEVEL=N | I – controls the level of detail in messages that are written to the SAS log. If the MSGLEVEL system option is set to N, the log displays notes, warnings, and error messages only. If MSGLEVEL is set to I, the log displays additional notes pertaining to index usage, merge processing, and sort utilities, along with standard notes, warnings, and error messages.

*More information on SAS options can be found here:

<http://www.sfu.ca/sasdoc/sashtml/lrcon/z0998454.htm>;

ODS LISTING CLOSE - Suppresses printing to the output window.

ODS RESULTS OFF - Suppresses printing to the results tab.

19. As a result of this sorting, the output file created in **Section 3.3.5** will contain genes ordered by descending absolute fold changes using *p*-values to break ordering ties in absolute fold change. Other sort options are available. For example, the “final” data set could also be sorted by *p*-value using the following code.

```
PROC SORT DATA = final;
  BY pvalue DESCENDING absoluteFC;
RUN;
```

Using this alternate code would yield an output file in **Section 3.3.5** containing genes ordered by ascending *p*-value with absoluteFC used to break ordering ties in *p*-value.

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References

1. Quackenbush, J. (2006) Microarray analysis and tumor classification. *N. Engl. J. Med.* **354**, 2463–2472.
2. Demmer, R. T., Behle, J. H., Wolf, D. L., Handfield, M., Kebschull, M., Celenti, R., Pavlidis, P., and Papapanou, P. N. (2008) Transcriptomes in healthy and diseased gingival tissues. *J. Periodontol.* **79**, 2112–2124.
3. Pavlidis, P. (2003) Using ANOVA for gene selection from microarray studies of the nervous system. *Methods.* **31**, 282–289.
4. Papapanou, P. N., Abbron, A., Verbitsky, M., Picosos, D., Yang, J., Qin, J., Fine, J. B., and Pavlidis, P. (2004) Gene expression signatures in chronic and aggressive periodontitis: a pilot study. *Eur. J. Oral Sci.* **112**, 216–223.
5. Delwiche, L. S., and Slaughter, S. J. (2003) *The little SAS book*, 3rd ed. SAS Institute Inc., Cary.
6. Lee, H. K., Braynen, W., Keshav, K., and Pavlidis, P. (2005) ErmineJ: tool for functional analysis of gene expression data sets. *BMC Bioinformat.* **6**, 269.
7. Draghici, S., Khatri, P., Bhavsar, P., Shah, A., Krawetz, S. A., and Tainsky, M. A. (2003) Onto-Tools, the toolkit of the modern biologist: Onto-Express, Onto-Compare, Onto-Design and Onto-Translate. *Nucleic Acids Res.* **31**, 3775–3781.
8. Khatri, P., Voichita, C., Kattan, K., Ansari, N., Khatri, A., Georgescu, C., Tarcam, A. L., and Draghici, S. (2007) Onto-Tools: new additions and improvements in 2006. *Nucleic Acids Res.* **35** (Web Server issue):W206–W211.
9. Storey, J. D., and Tibshirani, R. (2003) Statistical significance for genomewide studies. *Proc. Natl. Acad. Sci. USA.* **100**, 9440–9445.

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